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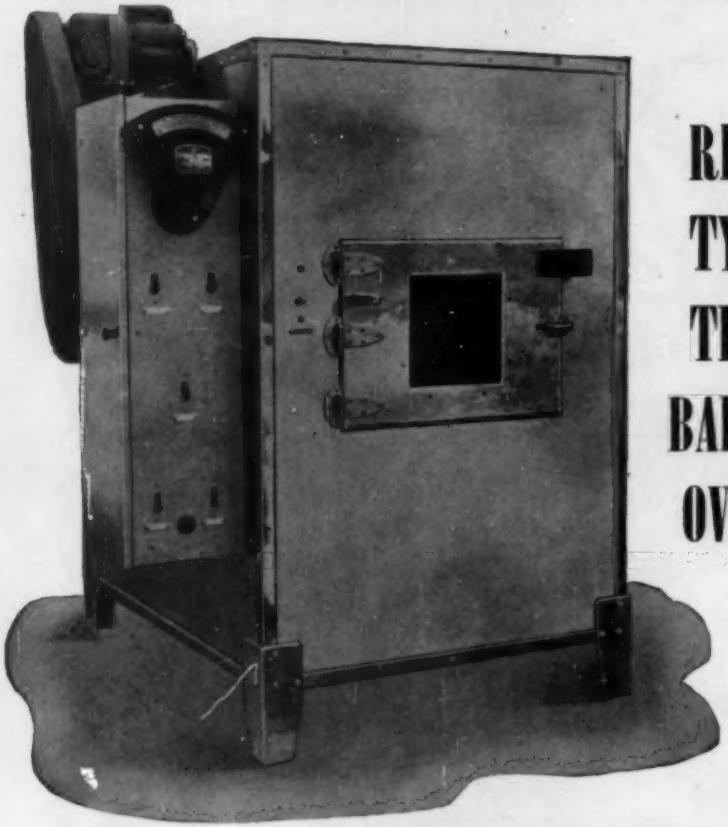

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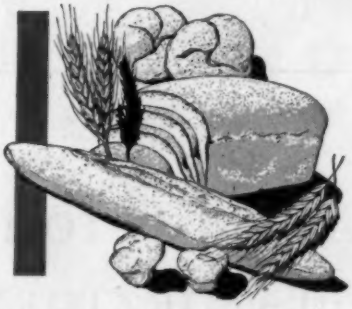
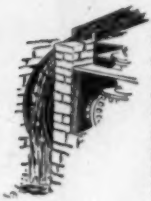


A Brief History of Milling and Baking by **SCIENCE WRITER**

CHAPTER VI. *Waterwheels and Millstones.*

THE STORY SO FAR

In preceding chapters we learned how men changed from hunting animals to cultivating grains for their food; how grain culture and milling improved as new grains and tools developed; how milling and baking left the home and became business; how the bread wheats and the arts of milling and baking spread across the world.



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Larger, more efficient millstones were developed as it was discovered that they could be turned by a source of power which exerted much more force than that supplied by men or animals. This new source was gravity in the form of swiftly-running water which was transmitted to grinding stones through waterwheels.

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In Roman times the architect Vitruvius described a waterwheel which powered a mill to grind grain.

Over the centuries waterwheels were improved. Gearing between the waterwheel and the millstones was introduced. Waterfalls and rapids were used as mill sites. In fact, cities often grew up around these places as Minneapolis around the Falls of St. Anthony. Mill dams were constructed to harness the power of the tides. Experimentation with the dressing of millstones brought about better milling. As the principles of hydraulics came to be understood, the direct drive mill gave way to more efficient types. Auxiliary drives were developed for hoisting the grain to the hopper and later for powering the sieves which bolted the meal.

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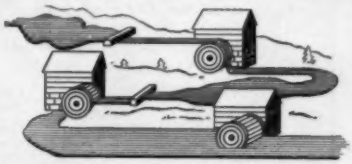

TODAY'S NUTRITION

Turbines evolved from waterwheels; cities grew from mill sites; and small, local millers and bakers expanded into nationwide organizations. As the business of milling grew so did the knowledge of wheat's nutritive values. Millers and bakers found that they could give the public the fine white flour and bread which it demanded and still supply important vitamins and minerals which are unavoidably lost during milling. They do this through enrichment which is the simple process of adding vitamins B_1, B_2, niacin plus iron and sometimes calcium to the flour as it is being milled. Result: better nutrition for countless millions of people.

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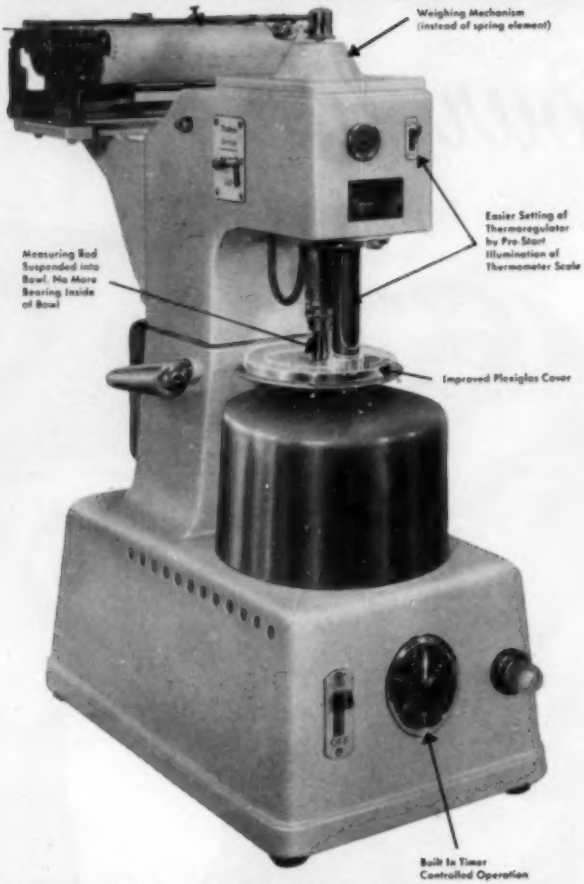
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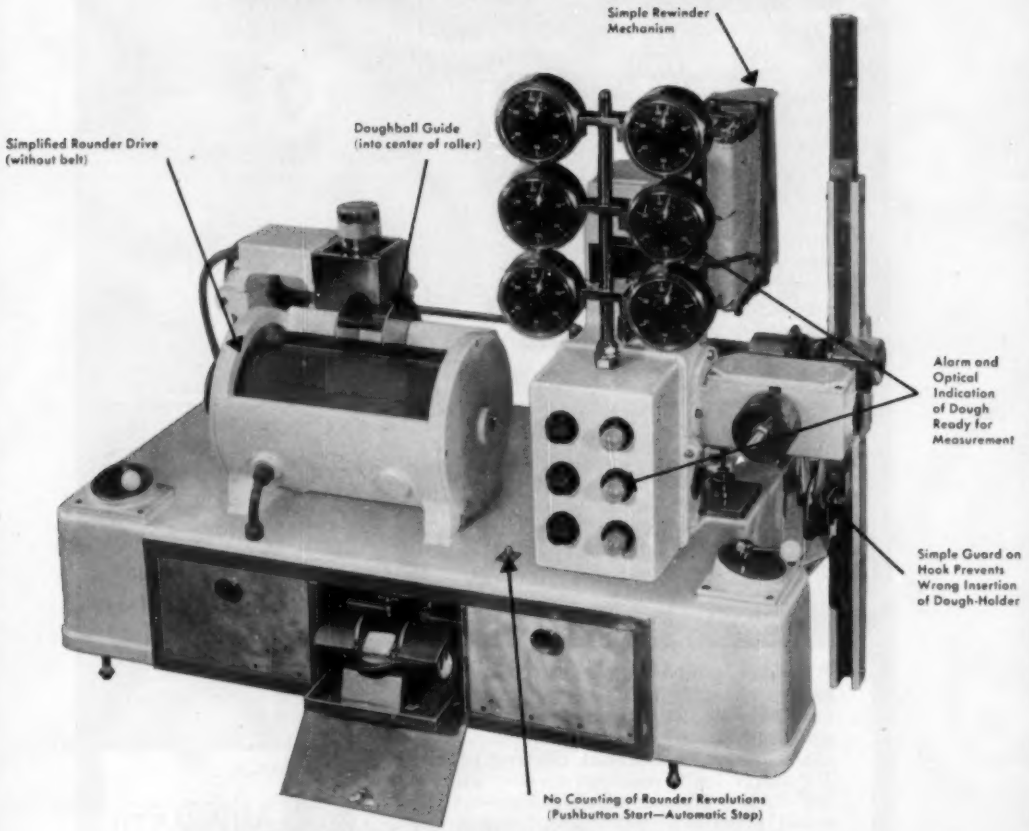
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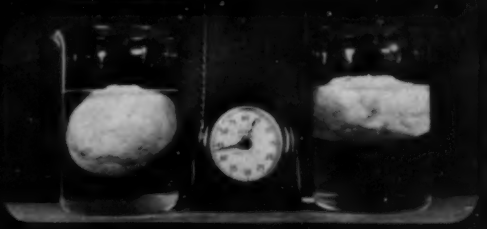

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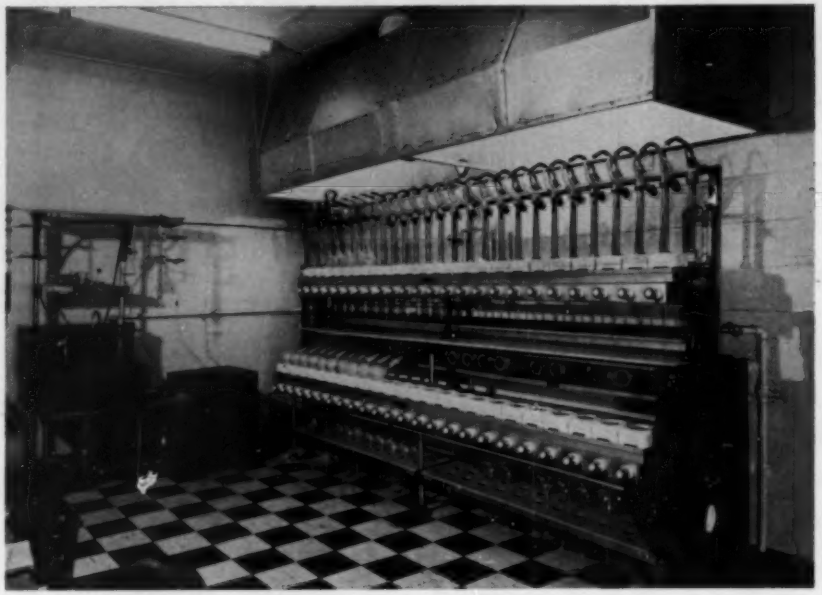

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CEREAL CHEMISTRY

VOL. 32 SEPTEMBER, 1955 NO. 5

EFFECT OF LEAF AND STEM RUST ON THE CONTENT AND DISTRIBUTION OF RIBOFLAVIN IN HARD RED SPRING WHEATS AT SUCCESSIVE STAGES OF KERNEL DEVELOPMENT[1]

A. HOFFER,[2] M. N. LEVINE,[3] AND W. F. GEDDES[4]

ABSTRACT

Riboflavin determinations were made on the grain, chaff, and straw from tillers harvested during kernel filling from plots of Thatcher and Marquis wheats variously infected with leaf and stem rust, respectively. Average kernel weights and moisture contents of entire tillers were determined as indices of progressive maturity.

Average kernel weight was adversely affected by severe rust infection, particularly stem rust. Transpiration rate was accelerated to a greater extent by stem rust than by leaf rust, even though the average seasonal stem rust load was only 48.7% as compared with a seasonal average load of 89.5% for leaf rust.

Starting in the early- or late-milk stage, the concentration of riboflavin in each plant fraction decreased with each succeeding stage of kernel formation, irrespective of variety or rust intensities. The total riboflavin content of the kernels of Thatcher wheat increased until the medium dough stage, and in those of Marquis until the soft dough stage. In the other fractions, as well as in the entire tiller, there was a more or less steady decrease in the total riboflavin content from early-milk to fully-ripe stages.

Because of its adverse effect on yield, severe leaf rust infection reduced the total riboflavin in the entire tiller by 23%, and severe stem rust by only 9%. The reduction of riboflavin in the kernels due to leaf rust was 34%, and that due to stem rust 38%.

Increases in the total riboflavin content of the kernels with their progressive development took place at a more rapid rate in the least severely rusted plants of either Thatcher or Marquis. The riboflavin content of the mature kernels ranged from 12 to 22% of that in the entire tiller, in the case of leaf rust; and from 13 to 19% in the case of stem rust.

A study begun in 1935 was extended in 1940 into a three-year WPA project which was initiated at the Minnesota Agricultural Experiment

[1] Manuscript received May 12, 1955. Paper No. 3382, Scientific Journal Series, Minnesota Agricultural Experiment Station, St. Paul 1, Minnesota. Joint contribution from the Department of Agricultural Biochemistry and the Department of Plant Pathology and Botany, Institute of Agriculture, University of Minnesota, in cooperation with the Cereal Crops Section, Field Crops Research Branch, Agricultural Research Service, U. S. Department of Agriculture. Grateful acknowledgment is made for valuable assistance rendered by the personnel of the Work Projects Administration, Official Project No. 165-1-71-124, sponsored by the University of Minnesota during the year 1941. This paper is based on a portion of the data contained in a thesis by Abram Hoffer, presented to the Graduate School of the University of Minnesota in partial fulfillment of the requirements for the Ph.D. degree, 1944.

[2] Director of Psychiatric Research, Department of Public Health, University Hospital, Saskatoon, Saskatchewan.

[3] Pathologist, Agricultural Research Service, U. S. Department of Agriculture.

[4] Professor of Agricultural Biochemistry, Institute of Agriculture, University of Minnesota, St. Paul.

Station to determine the effects of leaf rust (*Puccinia rubigo-vera tritici*) and stem rust (*Puccinia graminis tritici*) on the agronomic properties of spring wheat, the translocation of plant constituents into the developing kernel, and the chemical composition and industrial quality of the resulting grain. Extensive data were obtained, but only the findings on the distribution of thiamine in different fractions of essentially rust-free wheat plants at different stages of development (4) and the effects of leaf and stem rust on the phosphorus metabolism of the plants (6) have yet been published. The present paper deals with the effect of these fungi on the distribution of riboflavin in the various parts of the plant at different stages of maturity.

Several investigators have shown that the riboflavin content of forage crops decreases with maturity (7, 8, 9, 10, 11, 12, 14), but few studies have been reported on the riboflavin content of cereal grains at different stages of growth. Kohler (11) found that the riboflavin and thiamine contents of the oat plant were at a maximum at the jointing stage and thereafter markedly decreased. Robinson, Lynd, and Miles (13) studied the distribution of these vitamins in certain varieties of wheat, barley, and oats. The riboflavin content of wheat tillers decreased continuously until the plants ripened, when it became constant. In the case of barley and oats, there was an initial increase in the riboflavin content of the tillers, after which the amounts decreased until the grains were ripe. In the wheat and barley kernels there were initial increases in total riboflavin content, after which the amounts decreased to constant values when the grains were ripe. The total riboflavin content of the oat kernels continued to rise even after the grain was ripe. In all three cereals, the kernels contained less than one-half of the total riboflavin of the ripened tillers; for example, the ripened wheat kernels contained only 27 to 30% of the total riboflavin content of the tiller whereas the stems contained 43 to 55%.

It is well known that the influence of leaf and stem rust on the agronomic properties of wheat depends not only upon the extent of the infection but also upon the stage of development of the wheat plant when it is attacked. In these experiments the time and intensity of infection was controlled by artificial inoculation and by sulfur dusting. Riboflavin was determined on plants resulting from three treatments which gave light, medium, and severe rust infections.

Materials and Methods

Regulating Rust Infection on Plants. Thatcher was used for the study of the effect of leaf rust and Marquis for that of stem rust. The treatments used to induce the three degrees of leaf and stem rust in-

fection were as follows: 1) eight weekly applications of microfine sulfur dust at the rate of 50 lb. per acre onto the developing wheat plants from the tillering to the fruiting stages, with no artificial rust inoculations being used at any time; 2) eight weekly rust inoculations made during the identical developmental stages, each inoculation immediately followed by an application of sulfur dust only to the ground at the same rate as stated above; and 3) eight weekly rust inoculations in the manner just described, but no sulfur dusting whatever was applied under this method of treatment onto either the plants or the ground. During the season of 1941, when these treatments were used, Thatcher was entirely free from stem rust and Marquis had only a very small amount of leaf rust.

In the case of leaf rust on Thatcher, the severity of infection under the first method ranged from 11.4% in the pre-milk stage to 42.5% in the full-ripe stage, while the seasonal rust load during the kernel formation period averaged 31.7%; the corresponding leaf rust averages under the second method were 43.2, 93.2, and 70.5%; under the third method they were 72.6, 100.0, and 89.5%, respectively. The stem rust infections on Marquis were considerably less severe; thus, under the first method of treatment, the averages ranged from 0.8% in the pre-milk stage to 5.5% in the near-ripe stage, while the seasonal stem rust load amounted to 3.2%; under the second treatment, the corresponding averages were 6.5, 30.6, and 20.3%; under the third, 25.6, 66.9, and 48.6%, respectively.

Sampling and Preparation of Plants for Analysis. The methods used for obtaining the samples and preparing them for analysis have been described by Geddes and Levine (4) and need only be outlined here. Seven days after blossoming and at semiweekly intervals thereafter, a selected number of entire aerial tillers were collected and weighed, after which the heads were clipped off and weighed. These two fractions were then dried at approximately 60°C. and their dry weights recorded, after which the heads were threshed and the air-dry weights of the kernels and of the nonkernel portion determined. In this manner a total of three plant fractions—grain (kernels), chaff (glumes and rachides), and straw (stems and leaves)—were provided for the riboflavin determinations. Each plant fraction was ground in a Wiley laboratory mill to pass a sieve having 0.5-mm. openings and stored in air-tight containers.

Methods of Analysis. The moisture contents of the air-dried samples were determined by the 1-hour, 130°C. air-oven method described in *Cereal Laboratory Methods* (1). The moisture present in the entire tillers at each stage of maturity was computed from the weights of the

various fractions and of the moisture lost on air-drying and oven-drying.

The riboflavin determinations were made essentially according to the method of the Subcommittee on Riboflavin Assay described by Andrews (2, 3), but with some modifications reported by Hoffer, Alcock, and Geddes (5). The analyses were made in duplicate on different days and were recorded on the dry matter basis. As an additional index of maturity and also as a measure of the influence of rust infection on the filling of the kernels, the dry matter weights per 1000 kernels were determined. Five hundred air-dry kernels were counted and weighed in duplicate and the results computed to a dry-matter basis.

Results

Effect of Rust Infection on Average Kernel Weight and Water Loss at Successive Stages of Kernel Development. The kernel weights and moisture contents of the entire aerial tiller at successive stages of kernel

TABLE I

EFFECT OF MATURITY AND RUST INFECTION ON AVERAGE KERNEL WEIGHT AND RELATIVE MOISTURE CONTENT OF AERIAL TILLERS OF VARIOUSLY TREATED HARD RED SPRING WHEAT

Days after Blossoming	Successive Stages of Kernel Development	Treatment Results: 0 Rust Inoculations; Plants Sulfur-Dusted		8 Rust Inoculations; Ground Sulfur-Dusted		8 Rust Inoculations; No Sulfur Dusting	
		Weight per 1000 Kernels (dm basis)	Moisture Content of Aerial Tillers	Weight per 1000 Kernels (dm basis)	Moisture Content of Aerial Tillers	Weight per 1000 Kernels (dm basis)	Moisture Content of Aerial Tillers
		g.	%	g.	%	g.	%
	Thatcher	31.7% leaf rust		70.5% leaf rust		89.5% leaf rust	
7	Pre-milk	3.7	70.8	4.1	71.7	4.6	71.2
10	Early-milk	6.0	69.8	6.2	68.4	6.6	68.3
14	Late-milk	9.1	65.2	8.8	65.7	9.7	65.1
17	Soft-dough	12.5	64.0	11.4	64.0	12.5	62.6
21	Medium-dough	16.1	59.0	15.9	58.1	16.5	57.2
24	Hard-dough	19.9	52.2	17.7	48.7	18.1	48.8
28	Semi-ripe	20.1	39.6	16.8	33.0	17.7	26.2
31	Full-ripe	21.2	25.8	17.7	22.6	18.7	20.8
	Marquis	3.2% stem rust		20.3% stem rust		48.6% stem rust	
7	Pre-milk	6.0	68.4	6.5	67.2	5.9	67.3
10	Early-milk	9.9	66.0	9.9	65.6	9.7	65.6
14	Late-milk	14.2	61.2	14.5	60.6	13.9	59.7
17	Soft-dough	18.9	54.4	17.0	54.3	15.0	52.1
21	Medium-dough	23.3	46.8	17.7	43.0	16.9	35.4
24	Hard-dough	24.0	36.2	20.9	28.8	17.5	20.5
29	Near-ripe	24.4	22.6	20.5	20.2	18.2	19.3

formation in Thatcher and Marquis wheat in 1941 are recorded in Table I. The unfavorable effect of severe leaf rust infection on the average kernel weight of Thatcher first became apparent in the late-milk stage, where sulfur had been applied to the ground, and in the hard-dough stage, where no sulfur was used. The ultimate kernel weights of 21.2, 17.7, and 18.7 g. per 1000 with increasing rust infection on Thatcher wheat demonstrate that severe leaf rust infection materially reduces the average kernel weight. The effect of severe leaf rust infection (89.5%) on water loss became evident in the soft-dough stage; with less severe infection (70.5%), the effect was noted at the medium-dough stage. The maximum difference in the moisture content of the tillers from the three treatments of 13.4% was observed at the semi-ripe stage.

The influence of varying severities of stem rust infection on the kernel weight of Marquis wheat became clearly evident at the soft-dough stage. The effect of moderate infection on water loss became pronounced at the medium-dough stage, whereas severe infection appreciably decreased the moisture content of the tillers at the soft-dough stage. The maximum difference in the moisture content of the tillers attributable to varying degrees of rust infection of 15.7% occurred at the hard-dough stage.

Riboflavin Content of Thatcher Wheat under Three Intensities of Leaf Rust at Successive Stages of Kernel Development. The riboflavin content of Thatcher wheat is shown in Table II. On a unit weight basis, expressed as μg. per g. of dry matter, the riboflavin concentration in the grain (kernels) and other plant parts decreased very rapidly throughout the maturation period. Differences in the severity of leaf rust infection seemingly had little, if any, effect on the concentration of riboflavin in the several plant fractions or in the whole tiller.

The total riboflavin in the kernels increased progressively until the medium-dough stage (21 days after blossoming), after which a decline set in. The decline was the heaviest in the grain from the ground-dusted plots, which amounted to some 63%. Severe leaf rust infection appeared to favor a relatively high concentration of riboflavin in the grain during the pre-milk and early-milk stages, although the chaff and straw and the tiller as a whole showed progressive decreases in total riboflavin.

The lightly leaf rust-infected lot showed the greatest shift in the riboflavin from the straw to the developing kernels, rising from 3% in the pre-milk stage to 22% in the full-ripe stage.

In the most severe leaf rust epidemic, the proportion of riboflavin

in the young kernels averaged 6%; but in fully ripe kernels, the average was 19%.

The amounts of riboflavin in each fraction at any time represent the net result of any synthesis, translocation, and destruction that may have occurred. Since the riboflavin content of the entire aerial tiller markedly decreased with maturity, destruction of the vitamin

TABLE II

CONTENT AND DISTRIBUTION OF RIBOFLAVIN IN THATCHER WHEAT AT SUCCESSIVE STAGES OF KERNEL DEVELOPMENT

Days after Blossoming	Successive Stages of Kernel Development	Riboflavin Concentration (dm basis)				Total Riboflavin per Tiller				Distribution of Riboflavin		
		Grain	Chaff	Straw	Entire Aerial Tiller	Grain	Chaff	Straw	Entire Aerial Tiller	Grain	Chaff	Straw
		μg/g	μg/g	μg/g	μg/g	μg	μg	μg	μg	%	%	%
	No artificial leaf rust inoculations; weekly sulfur dusting of plants; average seasonal rust load 31.7%											
7	Pre-milk	6.8	7.6	9.4	8.9	0.4	1.9	10.0	12.3	3	16	81
10	Early-milk	5.4	8.2	9.5	8.9	0.6	2.1	9.4	12.1	5	17	78
14	Late-milk	3.1	7.2	7.6	6.7	0.7	1.8	8.0	10.5	7	17	76
17	Soft-dough	3.7	7.8	6.7	6.1	1.4	1.8	5.9	9.1	15	20	65
21	Medium-dough	3.0	7.0	6.5	5.5	1.5	1.6	5.6	8.7	17	19	64
24	Hard-dough	1.9	5.1	4.9	3.9	1.1	1.1	3.9	6.1	18	18	64
28	Semi-ripe	2.0	3.9	4.0	3.3	1.0	0.8	2.8	4.6	21	18	61
31	Full-ripe	1.7	3.0	4.1	3.0	1.1	0.7	2.9	4.7	22	15	63
	Continuous leaf rust inoculations; only sulfur dusting of ground; average seasonal rust load 70.5%											
7	Pre-milk	6.4	7.5	7.6	7.5	0.4	1.8	7.1	9.3	5	19	76
10	Early-milk	5.1	7.1	7.8	7.4	0.7	1.6	7.0	9.3	8	17	75
14	Late-milk	2.9	6.7	7.1	6.2	0.6	1.4	4.8	6.8	10	21	69
17	Soft-dough	3.0	6.6	6.5	5.7	0.9	1.4	5.2	7.5	12	19	69
21	Medium-dough	2.4	5.6	5.7	4.6	1.1	1.3	4.3	6.7	17	19	64
24	Hard-dough	1.8	4.3	4.6	3.6	0.8	0.9	3.0	4.7	17	18	65
28	Semi-ripe	1.3	2.6	4.1	3.0	0.5	0.5	2.6	3.6	15	13	72
31	Full-ripe	1.3	3.0	3.9	3.0	0.4	0.6	2.4	3.4	12	17	71
	Continuous leaf rust inoculations; no sulfur dusting whatever; average seasonal rust load 89.5%											
7	Pre-milk	6.5	7.1	7.6	7.4	0.5	1.8	7.1	9.4	6	19	75
10	Early-milk	5.2	7.3	7.4	7.1	0.8	1.6	6.2	8.6	9	18	73
14	Late-milk	2.9	6.9	7.6	6.6	0.7	1.4	6.2	8.3	8	17	75
17	Soft-dough	3.1	7.5	6.2	5.7	0.9	1.6	4.6	7.1	13	22	65
21	Medium-dough	2.3	6.2	5.9	4.8	1.0	1.3	4.1	6.4	15	20	65
24	Hard-dough	1.7	4.2	4.8	3.7	0.7	0.8	3.1	4.6	16	18	66
28	Semi-ripe	1.4	2.9	4.3	3.1	0.5	0.5	2.5	3.5	15	14	71
31	Full-ripe	1.6	3.0	4.0	3.0	0.7	0.6	2.3	3.6	19	17	64

greatly exceeded any synthesis that might have taken place. The progressive increases in the total riboflavin content of the grain until the medium-dough stage show that either synthesis or translocation or both have taken place. Severe leaf rust infection contributed to less synthesis or greater destruction of riboflavin in the tiller as a whole and in the straw and chaff fractions throughout the developmental period of the grain. The injurious effect of severe leaf rust infection on the total content of riboflavin in the kernel became evident during the late-milk and soft-dough stages of the grain.

TABLE III

CONTENT AND DISTRIBUTION OF RIBOFLAVIN IN MARQUIS WHEAT AT SUCCESSIVE STAGES OF KERNEL DEVELOPMENT

Days after Blossoming	Successive Stages of Kernel Development	Riboflavin Concentration (dm basis)				Total Riboflavin per Tiller				Distribution of Riboflavin		
		Grain	Chaff	Straw	Entire Aerial Tiller	Grain	Chaff	Straw	Entire Aerial Tiller	Grain	Chaff	Straw
		μg/g	μg/g	μg/g	μg/g	μg	μg	μg	μg	%	%	%
No artificial stem rust inoculations; weekly sulfur dusting of plants; average seasonal rust load 3.2%												
7	Pre-milk	5.3	7.7	8.8	8.3	0.7	2.7	11.5	14.9	5	18	77
10	Early-milk	5.6	9.0	7.3	7.4	1.4	2.9	9.2	13.5	10	22	68
14	Late-milk	3.7	7.6	7.6	6.7	1.4	2.1	7.9	11.4	12	18	70
17	Soft-dough	2.5	6.7	6.2	5.2	1.3	1.9	6.8	10.0	13	18	69
21	Medium-dough	1.9	4.6	6.0	4.4	1.1	1.3	6.2	8.6	13	15	72
24	Hard-dough	1.7	4.1	4.4	3.4	1.2	1.2	4.5	6.9	17	18	65
29	Near-ripe	1.6	3.7	4.1	3.1	1.1	1.1	3.9	6.1	19	18	63
Continuous stem rust inoculations; sulfur dusting of ground only; average seasonal rust load 20.3%												
7	Pre-milk	4.2	7.1	6.5	6.4	0.7	2.4	8.7	11.8	6	21	73
10	Early-milk	4.6	8.7	6.2	6.4	1.2	2.8	7.7	11.7	10	24	66
14	Late-milk	3.0	6.8	5.9	5.4	1.3	2.2	6.6	10.1	12	22	66
17	Soft-dough	2.3	5.6	5.6	4.7	1.1	1.5	5.4	8.0	14	19	67
21	Medium-dough	1.6	4.0	4.2	3.4	0.8	1.0	3.9	5.7	14	18	68
24	Hard-dough	1.6	3.2	3.8	3.0	0.9	0.9	3.5	5.3	17	17	66
29	Near-ripe	1.2	2.9	3.2	2.6	0.7	0.8	3.2	4.7	15	17	68
Continuous stem rust inoculations; no sulfur dusting whatever; average seasonal rust load 48.6%												
7	Pre-milk	5.5	7.4	7.5	7.3	0.8	2.2	8.8	11.8	7	19	74
10	Early-milk	4.9	9.4	6.8	7.0	1.2	2.7	7.4	11.3	10	24	66
14	Late-milk	3.0	6.9	6.5	5.8	1.1	2.0	6.5	9.6	12	20	68
17	Soft-dough	2.8	6.2	5.4	4.9	1.0	1.5	5.1	7.6	13	20	67
21	Medium-dough	1.6	4.2	5.8	4.6	0.6	1.0	5.3	6.9	9	14	77
24	Hard-dough	1.7	3.8	4.4	3.6	0.8	1.0	4.0	5.8	13	17	70
29	Near-ripe	1.6	3.7	4.0	3.3	0.7	1.0	3.9	5.6	13	18	69

Riboflavin Content of Marquis Wheat at Successive Stages of Kernel Development under Three Intensities of Stem Rust Infection. The results in Table III indicate that moderate as well as severe stem rust infection on Marquis wheat had a deleterious effect upon the riboflavin concentration of the entire tiller in the early stages of kernel formation, but this effect tended to disappear as the plant matured.

As the plants matured, there was a gradual decrease in the total riboflavin content of the entire tiller. In the grain, the total riboflavin content gradually increased up to about the late-milk stage and then declined. The near-ripe grain from plants showing 20.3 and 48.6% stem rust contained only 0.7 μg. of riboflavin per tiller, in contrast to 1.1 μg. for plants with only 3.2% rust infection.

With progressive development of the scantily rusted Marquis wheat, the relative amount of riboflavin in the grain increased from 5% in the pre-milk stage to 19% in the near-ripe stage. The grain from the moderately infected plants contained 15% of the total riboflavin of the entire tiller at the near-ripe stage; in those that were severely rust-infected the grain contained 13% of the total riboflavin at the near-ripe stage. On the other hand, the proportion of the total riboflavin in the straw increased as the intensity of the rust infection increased. The stem rust fungus, therefore, appeared to interfere with the translocation of riboflavin into the developing kernels of Marquis wheat.

Discussion

The effect of stem rust on the concentration, total content, and relative distribution of riboflavin in Marquis wheat varied considerably from that of leaf rust in Thatcher wheat. The intensity of stem rust on Marquis was less than that of leaf rust on Thatcher. Neither stem rust nor leaf rust exerted any appreciable effect upon the riboflavin concentration in the grain, chaff, or straw at the time the wheat approached or reached maturity. Differences were evident in the shifting proportion of riboflavin in the different plant parts. Severe leaf rust infection brought about a decrease of 23% in the riboflavin content of the entire mature tiller, whereas the reduction due to stem rust amounted to only 9%. In the ripe grain, on the other hand, severe stem rust infection caused a reduction in total riboflavin of 38%, while that due to very severe leaf rust infection was 34%. The reduction of total riboflavin in the chaff on account of severe leaf rust infection was 12%, in contrast to 10% caused by severe stem rust infection. While severe leaf rust infection caused a reduction of 21% of total riboflavin in the dead-ripe straw of Thatcher wheat, severe stem rust exerted no effect on the riboflavin content of the near-ripe

Marquis straw. Whatever translocation of riboflavin into the kernels may have taken place, it proceeded at a more rapid tempo in the least severely rusted plants of either Thatcher or Marquis.

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Literature Cited

1. American Association of Cereal Chemists. Cereal laboratory methods (4th ed.). The Association: St. Paul, Minn. (1941).
2. Andrews, J. S. A collaborative study of riboflavin assay methods. Cereal Chem. **20:** 3–23 (1943).
3. Andrews, J. S., Chairman. Report of 1942–43 Methods of Analysis sub-committee on riboflavin assay. Cereal Chem. **20:** 613–625 (1943).
4. Geddes, W. F., and Levine, M. N. The distribution of thiamin in the wheat plant at successive stages of kernel development. Cereal Chem. **19:** 547–552 (1942).
5. Hoffer, A., Alcock, A. W., and Geddes, W. F. Some factors affecting the determination of riboflavin by the fluorometric method. Cereal Chem. **21:** 515–523 (1944).
6. Hoffer, A., Geddes, W. F., and Levine, M. N. Effect of leaf and stem rust on the phosphorus metabolism of hard red spring wheat. Cereal Chem. **23:** 52–64 (1946).
7. Hunt, C. H., and Bethke, R. M. Riboflavin content of certain hays and grasses. J. Nutrition **20:** 175–180 (1940).
8. Hunt [C. H.], Bethke [R. M.], Wilder [O. H. M.], and Bell [R. M.]. Stage of maturity of hays as it affects their biological and chemical analyses. *In* Fifty-First Annual Report, Ohio Agricultural Experiment Station, pp. 89–90. Ohio Agr. Exp. Sta. Bull. 516 (Jan. 1933).
9. Hunt, C. H., and Krauss, W. E. The influence of the ration of the cow upon the vitamin B and vitamin G content of milk. J. Biol. Chem. **92:** 631–638 (1931).
10. Hunt, C. H., Record, P. R., Wilder, W., and Bethke, R. W. Factors influencing the vitamin-B and vitamin-G content of hays. Ohio Agr. Exp. Sta. Bimo. Bull. **18** (163): 104–106 (July-Aug. 1933).
11. Kohler, G. O. The effect of stage of growth on the chemistry of the grasses. J. Biol. Chem. **152:** 215–232 (1944).
12. Munsell, Hazel E. Vitamins and their occurrence in foods. Milbank Memorial Fund Quarterly **18:** 311–344 (1940).
13. Robinson, A. D., Lynd, L. E., and Miles, Barbara J. The distribution of thiamine and riboflavin in wheat, oats, and barley at successive stages of plant growth. Can. J. Research **B26:** 711–717 (1948).
14. Wilgus, H. S., Jr., Norris, L. C., and Heuser, G. F. The relative vitamin G content of alfalfa meal. Poultry Science **15:** 446–453 (1936).

SOME PROPERTIES OF FUNGAL LIPASES AND THEIR SIGNIFICANCE IN STORED GRAIN[1]

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ABSTRACT

Several properties of the lipases of wheat germ and two common storage molds were determined, using manometric or steam distillation and titrimetric procedures with mono-n-butyrin as the substrate. Differences in pH optima, temperature optima, the effect of ionic strength, stability to heat, and K_m values were greater between the lipases of the two molds than between the mold lipases and wheat germ lipase. Parachloromercuribenzoate and *o*-iodosobenzoate at 0.001 *M* concentrations inhibited wheat germ lipase 87% and 90%, lipase of *Aspergillus candidus* 17% and 12%, and a lipase of *Penicillium* 0% and 4%, respectively, thus providing a method for differentiating the mold lipases from those of wheat germ. Since lipase activity in moldy wheat was inhibited to a lower degree by *o*-iodosobenzoate than was the activity in nonmoldy wheat, the appearance of fat acidity in stored grain was attributed to fungal lipase activity. A nondialyzable, heat-labile inhibitor of the mold lipases was found to be present in water extracts of wheat germ.

Investigations of lipases in cereals and cereal products have been reviewed by Bradshaw (3), Geddes (8), Milner (14), and Sullivan (24). Relatively little attention had been given to the lipases of cereal grains until quite recently, although the increase of fat acidity in deteriorating grain has been known for some time. Singer and Hofstee (22, 23) have reported several properties of wheat germ lipase by use of manometric technics, but the importance of molds in grain deterioration, as pointed out by the researches of Geddes and his coworkers (10, 15, 16, 17), suggests that fungal lipase may be responsible for the observed fat acidity. Little is known of the nature of lipases produced by common storage molds, although some studies of fungal lipases have been made (6, 7, 9, 11, 18, 19, 20, 25). Dirks (5) has discussed the problem in detail. A study of the lipases of some common molds was undertaken with the hope that some property might be found which would differentiate them from wheat germ lipase and permit a conclusion regarding the relative importance of fungal lipases in stored grain.

Materials and Methods

Preparation of Wheat Germ Lipase. Commercial wheat germ was defatted by stirring 200-g. batches at least five times with three vol-

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umes of low-boiling petroleum ether (boiling range 35°–60°C.). The solvent was filtered off between washings on a large Büchner funnel. The dry defatted germ was then ground in a Wiley Mill to pass a 40-mesh sieve and stored at —10°C. Singer and Hofstee (22) reported that such dried preparations were stable for one year at —10°C.

Unless otherwise noted, 1 g. of the dry powder was homogenized briefly in ice-cold water with a Potter-Elvehjem homogenizer and the particulate matter centrifuged down. The supernatant was dialyzed 18 hours against cold (3°C.) distilled water, after which it was transferred to vials and frozen. The activity did not change appreciably with several thawings and refreezings for as long as 2 weeks. Although some activity was lost during dialysis, this treatment was deemed expedient in order to eliminate carbon dioxide retention in the manometric assay due to dialyzable buffer salts.

Preparation of Fungal Lipase. Several species of molds were isolated from samples of wheat by the use of the technic described by Christensen (4) for the quantitative determination of molds. Mold colonies were allowed to develop at room temperature. When the colonies were actively sporulating, spores were transferred from isolated colonies to poured plates of Czapek's solution agar (26). When well developed, the colonies were examined closely for contamination and tentatively identified. Pure colonies were transferred to slants of Czapek's solution agar and stored in the refrigerator after active growth had occurred. Mold species isolated and identified were *Aspergillus glaucus, A. candidus, A. versicolor, Rhizopus,* and a species of *Penicillium* which was probably *P. ochraceus.* All were found to produce an active lipase but only *A. candidus* and the *Penicillium* species were chosen for investigation to represent two different groups of common storage molds whose lipases may have different properties.

For the production of fungal lipase by the isolated mold species, the molds were allowed to grow on sterilized wheat bran to which a small amount of wheat germ oil was added to stimulate lipase synthesis. Slants of Czapek's solution agar were inoculated with spores of the various molds and allowed to incubate at room temperature until the colonies were heavy and actively sporulating. Twenty-five milliliters of sterile saline solution were introduced into each tube and agitated in order to get a suspension of spores. Ten milliliters of this suspension were added, by means of a sterile pipet, to previously sterilized 500-ml. Erlenmeyer flasks containing 30 g. wheat bran and 1 ml. wheat germ oil and stoppered with cotton. The moisture added in this manner was enough to raise the total moisture content to about 25%. At the same time as the bran was inoculated, some of each spore suspension was

streaked on to Czapek's solution agar. In this way it was possible to identify and discard any flasks whose contents showed evidence of contamination. Flasks of sterile bran treated with sterile saline were also carried along as controls. No lipase activity could be detected in these noninoculated bran samples.

After 2 weeks at room temperature, the bran had become very moldy and was removed from the flasks for air drying. The samples were defatted with petroleum ether, ground and stored at —10°C. in the same manner as that described for wheat germ. Extraction procedures were also the same as that for wheat germ except that the lipase activity in the mold bran extracts was generally so much higher than in the wheat germ extracts that a twentyfold dilution was necessary to give a working solution with a convenient level of activity.

Procedure for Manometric Assay of Lipase Activity. The manometric procedure used was based on that described by Singer and Hofstee (22). An appropriate amount of enzyme solution (usually 1 ml.) was placed in the main compartment of a conventional Warburg vessel, preferably without a center well, followed by sodium bicarbonate solution of sufficient concentration to give the desired pH (28) and other additions. The mono-n-butyrin substrate (Eastman), purified by distillation under high vacuum, was dissolved in sodium bicarbonate solution of the same molarity as that in the main compartment and placed in the sidearm. After gassing 5 minutes with a 5% carbon dioxide-95% nitrogen gas mixture, the flasks were allowed to remain 15 minutes in the constant-temperature water bath (35° or 38°C., depending upon the enzyme source) for temperature equilibration. After the contents of the sidearm had been tipped into the main compartment, the evolution of carbon dioxide was followed by taking manometer readings at 5- or 10-minute intervals from 0 to 40 minutes. The manometers were shaken at 150 oscillations per minute during this time. Corrected manometer readings were plotted against time, and the initial rate of carbon dioxide evolution was used as a measure of lipase activity. Corrections were made for carbon dioxide retention where it was found necessary.

Steam Distillation Procedure for Lipase Assay. Singer and Hofstee (22) also outlined a steam distillation procedure in which mono-n-butyrin fulfilled the substrate requirement. Adequate buffering of the reaction mixture was obtained by the use of MacIlvain's phosphate-citrate buffer (0.2 *M* disodium phosphate and 0.1 *M* citric acid mixed in varying proportions to give pH values over the range of pH 4 to 8). Briefly, the procedure involved the addition to test tubes of 2 ml. buffer solution, an appropriate volume of enzyme solution, and

enough water to make 5 ml. total volume after addition of substrate. The contents were brought to 38°C. in a water bath and the preheated substrate solution was added. At the end of 30 minutes, the pH was determined with a glass electrode and enough 10% metaphosphoric acid added to bring the pH to 3.0. Water was added to give a total volume of 7 ml., the precipitated protein was centrifuged down, and a 5-ml. aliquot was taken for steam distillation. The first 100 ml. of distillate were titrated with 0.01 *N* sodium hydroxide solution using the cresol red-thymol blue indicator of Kleinzeller and Trim (12). Results were recorded as ml. 0.01 *N* sodium hydroxide solution required to neutralize the butyric acid released. Complete recovery of butyric acid was obtained by this procedure.

Continued hydrolysis of the substrate at pH 3.0 made it necessary to carry out the distillation as quickly as possible after stopping the enzymic reaction with metaphosphoric acid. In experiments using this procedure, the contents of each reaction tube were treated according to a strict time schedule, so that the time lag between the end of the enzymatic reaction and the beginning of steam distillation was constant and at a minimum. Substrate blanks (no enzyme) were carried with each assay at each pH level used, because the rate of blank hydrolysis of mono-n-butyrin was found to increase sharply when the pH of the reaction mixture was raised above 7.5. All values reported by use of this method have been corrected for nonenzymatic substrate hydrolysis determined simultaneously. Enzyme blanks (no substrate) were insignificant.

This procedure is tedious and time-consuming, and initial reaction rates cannot be obtained readily. Nevertheless, it has the advantage of flexibility in regard to pH ranges and buffers, and provides better conditions for titration than are found in many titrimetric lipase assay methods. Despite the inconveniences of the Warburg apparatus, however, the manometric method is well suited for obtaining accurate kinetic data, provided the pH required is within the range of the bicarbonate buffers.

Properties of Fungal and Wheat Germ Lipases

Enzyme Concentration vs. Activity. A straight line relationship was found to exist between enzyme concentration and activity for each of the lipases under study (Fig. 1).

Determination of pH Optima. In order to determine the optimum pH for activity of the different lipases, the steam distillation procedure described previously was used. The optimum determined for wheat germ lipase was pH 7.2, for lipase of *Aspergillus candidus,* 6.7,

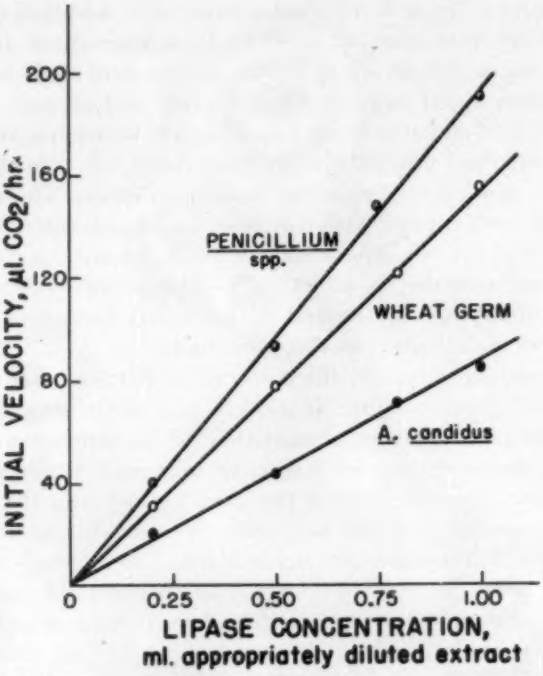

Fig. 1. Effect of enzyme concentration on activity of lipases from various sources. Mono-n-butyrin, 0.170 M; bicarbonate buffer, pH 7.4, 38°C.

and for the lipase of *Penicillium*, 6.3. Although activities of the various preparations used were adjusted to be at about the same level according to the manometric procedure, the Penicillium preparation had a surprisingly high activity in the steam distillation procedure. This will be discussed later.

Since all the pH optima found were within the range of bicarbonate buffers, it was deemed advisable to check these values with the manometric assay. The wheat germ lipase optimum at pH 7.3 agreed well with the previous results and the lipase of *A. candidus* at pH 7.0–7.2 was not too far afield. However, the lipase of *Penicillium* gave entirely unexpected results, with its highest activity at pH 8.0. This shift in pH optimum with the different procedure seemed rather large to be due to the change of buffer alone, and appeared to have been influenced by other factors. Ionic strength was suspected as a possible reason because, when bicarbonate concentration was adjusted to change the pH, the ionic strength was also changed.

Accordingly, a check of pH optima for the lipases of *A. candidus* and *Penicillium* was made, keeping the ionic strength constant with

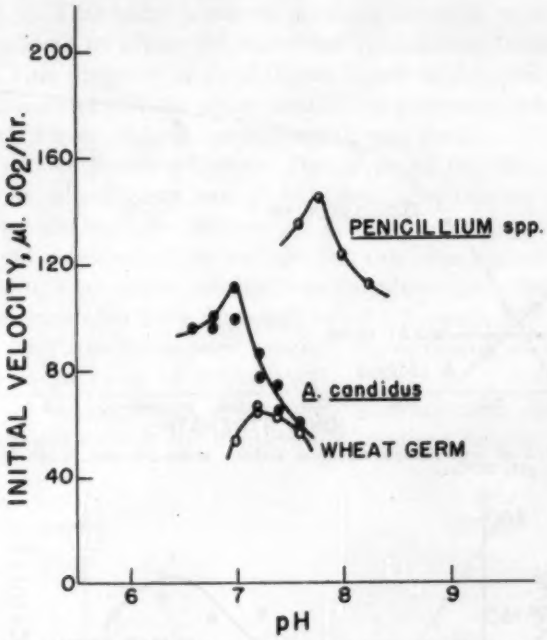

Fig. 2. Effect of pH on lipase activity at constant ionic strength as determined by the manometric procedure. Mono-n-butyrin, 0.166 M; bicarbonate buffer, 38°C.; ionic strength, 0.100.

appropriate amounts of sodium chloride. Results of these tests are shown in Fig. 2. The pH optimum for Penicillium lipase at an ionic strength of 0.10 was at pH 7.7–7.8, a value still considerably higher than that obtained in the steam distillation procedure using a phosphate-citrate buffer. Although ionic strength has some effect (to be presented in the next section), there are undoubtedly other factors influencing the shift in pH optimum. At the same ionic strength ($\mu = 0.10$), the lipase of *A. candidus* has an optimum of pH 6.8, which agrees almost exactly with that found by the steam distillation procedure.

Ionic Strength Effects. The foregoing results indicated that ionic strength might have different effects on the different lipases being studied. Each lipase was therefore tested at its optimum pH at different ionic strengths. Adjustments in ionic strength were made by adding appropriate amounts of sodium chloride. The results, in Fig. 3, show that the activity of the lipase of *A. candidus* was depressed by increasing ionic strength, activity of the Penicillium lipase was stimulated, while the wheat germ lipase was unaffected by changes in sodium

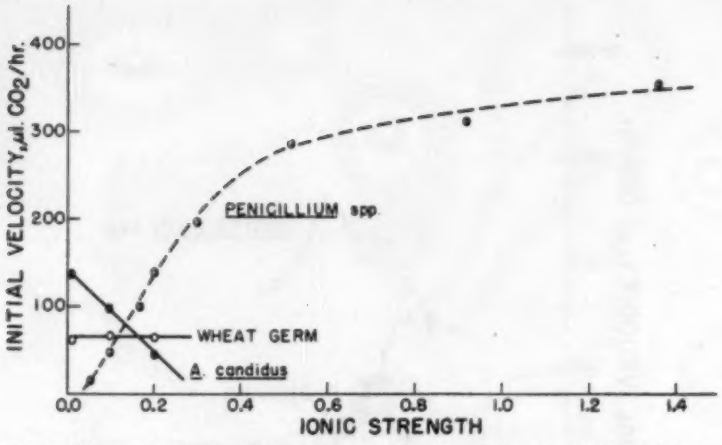

Fig. 3. Effect of ionic strength on lipase activity. Mono-n-butyrin, 0.167 M; bicarbonate buffer, optimum pH; 38°C.

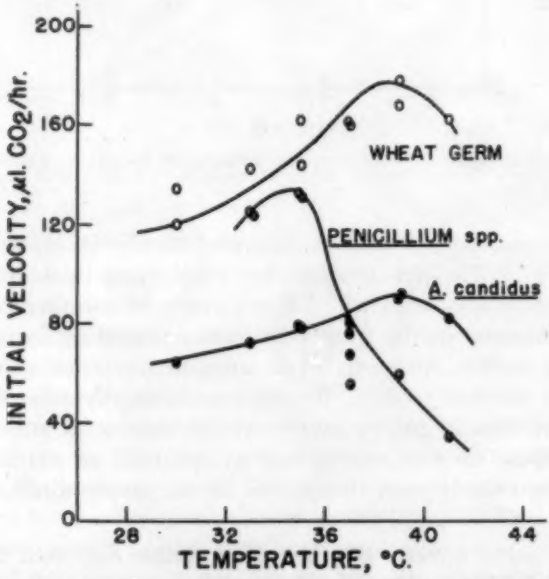

Fig. 4. Effect of temperature on lipase activity. Mono-n-butyrin, 0.169 M; bicarbonate buffer, pH 7.5.

chloride concentration. The reason for this difference is not apparent, but further investigations of this phenomenon were beyond the scope of this paper.

It is interesting to note here that Thibodeau and Macy (25) found a maximum activity for lipase of *P. roqueforti* at salt concentrations

of about 4%. This corresponds to an ionic strength of 0.7 which is remarkably close to where the curve for Penicillium lipase in Fig. 3 levels off. This property of Penicillium lipase might also explain its high relative activity in the steam distillation procedure where a phosphate-citrate buffer of high ionic strength was used.

Apparent Temperature Optima. Data showing the effect of temperature on the wheat germ and mold lipase activities are plotted in Fig. 4. Since the sodium bicarbonate concentration was changed to keep the pH constant, some sodium chloride was added to control ionic strength. The curves indicate that the wheat germ lipase and the lipase of *A. candidus* have apparent optima between 37° and 40°C. under the assay conditions used, whereas the optimum of Penicillium lipase falls in the range of 30° to 35°C.

Heat Stability. A better property to measure than the apparent optimum temperature is the temperature required for inactivation of

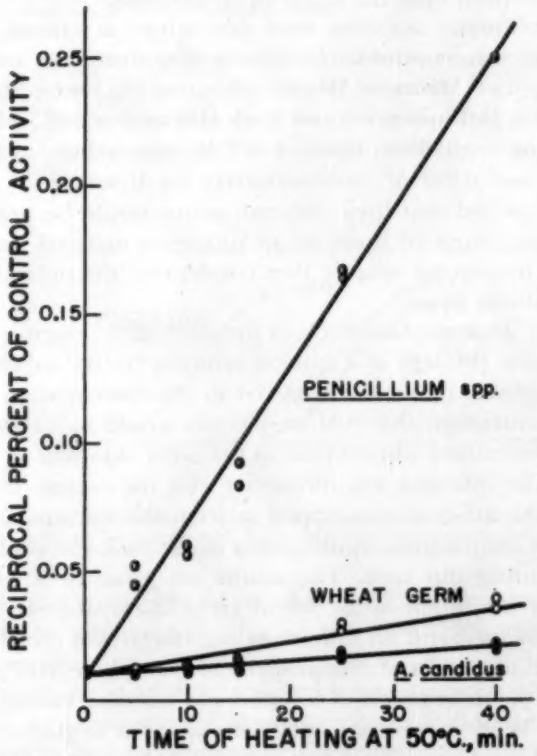

Fig. 5. Effect of heating at 50°C. on reciprocal lipase activities. Mono-n-butyrin, 0.169 M; bicarbonate buffer, pH 7.5; 38°C. for lipases of wheat germ and A. candidus; 35°C. for Penicillium lipase.

a measured amount of enzyme. Dialyzed extracts of each lipase source were brought rapidly to 50°C. in a water bath. At 5, 10, 15, 25, and 40 minutes, 3-ml. aliquots were withdrawn and cooled immediately in a freezing mixture. These samples together with the unheated control, were assayed by the manometric procedure under conditions optimum for each enzyme.

Singer and Hofstee's (23) data for a similar experiment with wheat germ lipase agreed well with the results obtained. These investigators pointed out that, unlike most enzyme systems, wheat germ lipase has a second-order reaction of heat inactivation. Accordingly, reciprocal activities for each of the lipases investigated were plotted against time of heating. The results are presented in Fig. 5. The straight lines obtained show that the second-order inactivation applies also to the fungal lipase. It is obvious that the Penicillium lipase was most sensitive to heat and that wheat germ lipase was somewhat more susceptible to heat inactivation than the lipase of *A. candidus*.

K_m Value. Enzyme activities were determined at various substrate concentrations using optimum conditions of temperature and pH for each enzyme. The Michaelis-Menten constants (K_m) were then calculated according to Lineweaver and Burk (13) to give 0.013 *M* mono-n-butyrin for the Penicillium lipase, 0.042 *M* mono-n-butyrin for wheat germ lipase, and 0.099 *M* mono-n-butyrin for lipase of *A. candidus*. It was not expected that these determinations would be practical for identifying the source of lipase in an unknown material, but the results proved interesting because they emphasized the different nature of the Penicillium lipase.

Inhibitors. To study the effects of the sulfhydryl reagents, *p*-chloromercuribenzoate (PCMB) and *o*-iodosobenzoate (IOB), on wheat germ and fungal lipases, they were dissolved in alkaline solution (pH 9.0) in such concentrations that 0.01 to 0.05 ml. would suffice to give the desired concentrations when added to the other solutions in the Warburg flask. The inhibitor was in contact with the enzyme for 30 minutes before the substrate was tipped in from the sidearm. Gassing of the flasks and temperature equilibration of the flask and contents were completed during this time. The results are given in Table I. The much greater inhibition of the wheat germ lipase suggests that these inhibitors may be useful for differentiating fungal and cereal enzymes.

The inhibitory effect of diisopropylfluorophosphate (DFP), reported to be one of the most powerful inhibitors of esterases known (29), was also studied. With enzyme concentrations adjusted to give activities of about 200 μl. of carbon dioxide per hour, flasks were prepared with and without DFP in the main compartment. The DFP was freshly pre-

TABLE I

INHIBITORY EFFECT OF *p*-CHLOROMERCURIBENZOATE (PCMB) AND *o*-IODOSOBENZOATE (IOB)

INHIBITOR	CONCENTRATION	LIPASE ACTIVITY[a]			INHIBITION		
		Wheat Germ	*Aspergillus candidus*	*Penicillium*	Wheat Germ	*Aspergillus candidus*	*Penicillium*
	M	$\mu l\ CO_2/hr$	$\mu l\ CO_2/hr$	$\mu l\ CO_2/hr$	%	%	%
PCMB	None	83	46	200	0	0	0
	0.0005	14	49	206	83	0	0
	0.0010	11	38	200	87	17	0
	0.0020	7	24	200	92	48	0
IOB	None	181	188	222	0	0	0
	0.0005	47	178	222	74	5	0
	0.0010	36	166	214	80	12	4
	0.0020	34	161	209	81	14	6

[a] Mono-n-butyrin, 0.170 M; bicarbonate buffer, pH 7.5; 38°C. for lipases of wheat germ and Aspergillus candidus; 35°C. for Penicillium lipase.

pared in sodium bicarbonate solution (1). Thirty minutes were allowed after the inhibitor was added before the substrate was tipped in and the reaction begun. Results are shown in Fig. 6 where the percent of inhibition is plotted against the negative logarithm (pI) of the in-

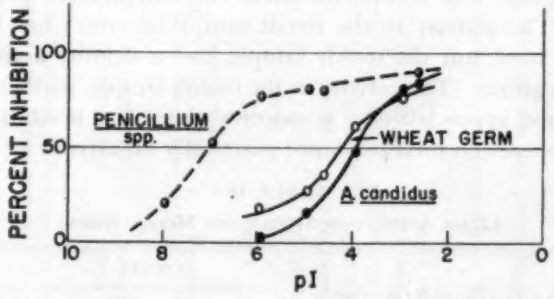

Fig. 6. Inhibition of lipases by diisopropylfluorophosphate (pI = negative logarithm of inhibitor concentration). Mono-n-butyrin, 0.169 M; bicarbonate buffer, pH 7.5; 38°C. for lipases of wheat germ and A. candidus; 35°C. for Penicillium lipase.

hibitor concentration. The figure used to express the susceptibility of the enzyme to inhibition is pI_{50}, or the pI at 50% inhibition. For wheat germ lipase, this became 4.3, and for *A. candidus*, 3.9. The Penicillium lipase, however, was much more susceptible, having a pI_{50} of 7.1. As a consequence, it must be concluded that the action of DFP cannot be used to differentiate the fungal lipases from the wheat germ lipase.

Lipase Activity in Stored Wheat

Lipolytic Activity in Moldy and Nonmoldy Wheat. Two samples of wheat were obtained from the same original lot of white wheat which was free from any detectable internal infestation. For certain respiration experiments, one sample had been stored without surface-sterilization with a sodium hypochlorite solution. Both carried 21% moisture during the storage period of 19 days at 35°C. and were aerated at the equilibrium relative humidity. The unsterilized wheat was visibly moldy and had a musty odor.

The two samples were ground and defatted with two portions of petroleum ether and kept in the refrigerator until assayed. Extraction was carried out by homogenizing 1.5 g. meal in 5 ml. of cold distilled water. The mixture was allowed to stand 30 minutes in the cold with occasional stirring and then centrifuged strongly to obtain a clear, homogeneous solution. The moldy sample gave a much browner extract than the sterile one.

Using mono-n-butyrin as the substrate, 1 ml. of extract was added in 3 ml. total volume in the Warburg flasks for manometric assay at pH 7.4 and a temperature of 38°C. Carbon dioxide retentions were determined for each sample and the activities measured in the absence and presence of the sufhydryl reagent, *o*-iodosobenzoate (IOB), at different levels. The results for these two samples are presented in Table II. The activity in the sterile sample was very low under the conditions used, but the moldy sample had a definite activity about eight times greater. The activity in the moldy sample, furthermore, was not inhibited appreciably by a concentration of as much as 0.002 *M* IOB in the reaction mixture. Since practically no activity in the moldy

TABLE II

LIPASE ACTIVITY IN STERILE AND MOLDY WHEAT

SAMPLE	MOLD COUNT	RETENTION OF CO_2	ACTIVITY			INHIBITION
			Concentration of *o*-Iodosobenzoate			
			None	0.001 *M*	0.002 *M*	
	molds/g	%	*µl CO_2/hr (corr.)*[a]	*µl CO_2/hr (corr.)*[a]	*µl CO_2/hr (corr.)*[a]	%
Nonsterile	88,800,000[b]	17.8	a. 120	110	..	8
			b. 112	...	106	5
Surface-sterilized	0	10.9	a. 16	18	...	0
			b. 14	...	12	14

[a] pH 7.4, 38°C., 0.170 M mono-n-butyrin substrate.
[b] Primarily Aspergillus flavus and A. ochraceus.

TABLE III

LIPASE ACTIVITY IN STORAGE SAMPLES OF HENRY WHEAT

SAMPLE NO.	CO_2 IN ATMOSPHERE[a]	MOLD COUNT[b]	FAT ACIDITY (dry basis)	LIPASE ACTIVITY[c]			INHIBITION	
				Concentration of *o*-Iodosobenzoate			Concentration of *o*-Iodosobenzoate	
				None	0.002 *M*	0.004 *M*	0.002 *M*	0.004 *M*
	%	*molds/g*	*mg KOH/ 100 g wheat*	*μl CO_2/hr*	*μl CO_2/hr*	*μl CO_2/hr*	%	%
0[d]		1,500	18.3	43	31	26	28	39
1	<1.0	5,500	61.0	24	22	23	8	4
2	4.0	13,400,000	66.1	36	35	33	3	8
3	6.9	13,650,000	62.0	34	28	26	17	23
4	9.6	14,950,000	60.0	42	39	37	7	12
5	12.2	13,000,000	62.0	37	30	28	18	24
6	14.3	16,900,000	69.2	43	37	36	14	16
7[e]		1,500	18.3	42	29	23	31	45

[a] With the exception of sample 1, the oxygen content of the storage atmosphere was kept constant at about 22%. The atmospheric oxygen for sample 1 was about 0.80%.
[b] All Aspergillus glaucus.
[c] Average of two or more determinations; control, five determinations; pH 7.4, 38°C., 0.172 M mono-n-butyrin substrate.
[d] Original control.
[e] Humidified control.

sample was inhibited by the IOB, it is unlikely that any cereal lipase activity remained in the moldy sample.

Table III presents further data concerning the lipase activity of Henry wheat samples[4] before and after storage at 17.6% moisture (85% relative humidity) under atmospheres containing various proportions of carbon dioxide. After storage for 15 days at 35°C., each sample was air-dried and ground to pass a 40-mesh sieve. Fat acidity was determined according to the procedure outlined in *Cereal Laboratory Methods* (2). Lipase activity was determined by the manometric method already described, except that 2 g. of the defatted sample were homogenized in 10 ml. of ice-water.

The results in Table III reveal that, although the fat acidity was considerably higher, the moldy samples had somewhat less lipase activity than the controls. However, since a higher proportion of the activity in the control samples was inhibited by IOB, it is possible that the lipase activity in the moldy samples included more of fungal origin than that in the nonmoldy ones.

TABLE IV

EFFECT OF VARIOUS LEVELS OF *o*-IODOSOBENZOATE ON LIPASE ACTIVITY IN NONMOLDY AND MOLDY WHEAT

WHEAT SAMPLE	*o*-IODOSOBENZOATE CONCENTRATION	ACTIVITY	INHIBITION
	M	$\mu l\ CO_2/hr$ [a]	%
0–Nonmoldy	0.000	54.4	0.0
	0.004	41.2	24.3
	0.010	32.4	40.4
	0.020	5.0	90.8
6–Moldy	0.000	35.8	0.0
	0.004	33.6	6.1
	0.010	31.2	12.8

[a] pH 7.4, 38°C., 0.172 M mono-n-butyrin substrate.

The effects of varying levels of IOB on the lipase activity of a nonmoldy and an exceptionally moldy wheat sample are shown in Table IV. Concentrations of inhibitor which very strongly inactivated the nonmoldy whole wheat lipases had much less effect on the lipases in the moldy samples. Since lower inhibitor concentrations inhibited the partially purified lipase of wheat germ much more strongly (see Table I), it is likely that the higher proportion of protein in crude whole

[4] Henry wheat is a hard red spring wheat of poor milling and baking quality. The lot of wheat used here was grown in Michigan in 1950. The authors are indebted to Mr. Brian Hummel of the Department of Agricultural Biochemistry, University of Minnesota, for providing the samples of wheat studied in these experiments.

wheat extracts may react with part of the inhibitor and make it less effective. In this regard, Singer (21) reported that the concentration of mercurial necessary for complete inactivation of wheat germ lipase depended on the stage of purity of the enzyme and the amount of protein present.

Inhibition of Lipase Activity by Extracts of Fungal and Wheat Germ Lipase Preparations. Inhibition or destruction of lipases in moldy and nonmoldy wheat with IOB indicated that inhibition of the cereal lipases may have occurred, inasmuch as a smaller proportion of the activity was inhibited by IOB in the moldy samples even though the total activity was less than in the nonmoldy samples. Also, there was the possibility that some factor in water extracts of wheat may inhibit the fungal lipase activity. The presence of an inhibitory factor or factors is shown by the results in Table V. These data indicate

TABLE V

MUTUAL INHIBITION OF LIPASE ACTIVITY IN EXTRACTS OF WHEAT GERM AND MOLD BRAN (*Aspergillus candidus*)

SOURCE OF ENZYME	VOLUME OF EXTRACT[a]	ACTIVITY	LOSS IN ACTIVITY
	ml.	$\mu l\ CO_2/hr$ *(corr.)*[b]	%
Wheat germ	0.5	64	...
Mold bran	0.5	312	...
Sum		376	
50% Wheat germ, 50% Mold bran	1.0	198	47
Wheat germ Mold bran	0.5 0.5	208	45

[a] 1 g. powdered enzyme preparation extracted with 20 ml. ice-water.
[b] pH 7.4, 38°C., 0.171 M mono-n-butyrin.

that extracts of wheat germ must have inhibited the activity of the mold lipase, because the loss in activity is greater than the total activity of the wheat germ extract alone.

Table VI gives results of experiments in which the activities of mixed extracts before and after dialysis were determined in the presence of IOB. The loss of activity in mixed extracts was again noted and could be ascribed to an inhibition of fungal lipase activity, since the absolute loss of activity with IOB was nearly the same for the mixture as for the wheat germ lipase alone. Doubling the concentration of mold bran extract to give twice the lipase activity shows that the assay conditions were not limiting. Higher concentrations of the inhibitor resulted in increased losses of activity in the mixed extracts. These data give evidence that a nondialyzable factor in the wheat germ ex-

TABLE VI

INHIBITORY EFFECT OF *o*-IODOSOBENZOATE ON LIPASE ACTIVITY IN EXTRACTS OF WHEAT GERM AND MOLD BRAN (*Aspergillus candidus*) AND MIXTURES THEREOF

ENZYME SOURCE	EXTRACT[a]	VOLUME USED	ACTIVITY		INHIBITION
			Concentration of IOB		
			None	0.002 M	
		ml.	*μl CO_2/hr (corr.)*[b]	*μl CO_2/hr (corr.)*[b]	%
Wheat germ	1/20	0.5	60	14	77
Mold bran	1/100	0.5	74	62	16
Mold bran	1/100	1.0	146	...	...
Wheat germ Mold bran	1/20 1/100	0.5 0.5	84	48	43
Wheat germ	1/10[c]	0.5	55	20	64
Mold bran	1/50[c]	0.5	115	116	0
Wheat germ Mold bran	1/10[c] 1/50[c]	0.5 0.5	104	67	36

[a] Extracts indicate ratio between g. powdered enzyme preparation and ml. ice-water used to extract.
[b] pH 7.4, 38°C., 0.170 M mono-n-butyrin.
[c] Dialyzed.

TABLE VII

EFFECT OF HEATING ON THE INHIBITORY ACTION OF EXTRACTS OF WHEAT GERM AND MOLD BRAN (*Aspergillus candidus*)

ENZYME SOURCE	EXTRACT[a]	VOLUME USED	LIPASE ACTIVITY		LOSS IN ACTIVITY
			Found	Expected	
		ml.	*μl CO_2/hr*[b]	*μl CO_2/hr*[b]	%
Wheat Germ	1/20	0.5	61	...	...
Wheat Germ	1/20, heated	1.0	5	...	...
Mold Bran	1/100	0.5	60	...	...
Mold Bran	1/100, heated	1.0	46	...	...
Wheat germ Mold bran	1/20 1/100, heated	0.5 0.5	84	84	0
Wheat germ Mold bran	1/20 1/100, heated	0.5 1.0	102	107	5
Mold bran Wheat germ	1/100 1/20, heated	0.5 0.5	52	63	17
Mold bran Wheat germ	1/100 1/20, heated	0.5 1.0	50	65	23

[a] Figures indicate ratio of g. powdered enzyme preparation to ml. ice-water. Heating was done at pH 6.6 in boiling water for 10 minutes.
[b] pH 7.4, 38°C., 0.170 M mono-n-butyrin.

tract was inhibiting fungal lipase.

Table VII summarizes the effect of heat on the inhibitory activity of extracts of both wheat germ and mold bran. No inhibitor to wheat germ lipase was present in heated mold bran extracts. Heating in boil-

ing water for 10 minutes, however, reduced but did not eliminate the inhibitor of mold lipase present in the wheat germ extract. The inhibitory factor or factors appear to be partially heat-labile under these conditions. It is interesting to note that a portion of the mold lipase activity is relatively heat-stable. The fact that this heat-stable activity is of the same magnitude as the residual mold lipase activity in mixtures of mold bran and wheat germ extracts (Table VI) suggests that the heat-stable mold lipase fraction is not inhibited by wheat germ extracts.

Further experiments (5) have shown that the inhibitory factor or factors in wheat germ extracts are not precipitable by trichloracetic acid.

Discussion

Though a comparison of properties of lipases of wheat germ, *A. candidus,* and a *Penicillium* species revealed differences, none could be used for differentiating fungal from cereal lipases except the inhibitory activity of sulfhydryl reagents. It is significant that both fungal lipases, though widely different in other properties, were not susceptible to inhibition by low concentrations of sulfhydryl reagents. In this regard Fiore and Nord (6) have found the lipase of *Fusarium* to be a true lipase but not susceptible to inactivation by sulfhydryl reagents. Whether other mold species react similarly remains to be seen, but on the basis of these results, it was possible to draw conclusions from studies of mold-free and moldy wheat regarding the relative significance of cereal and mold lipases in grain storage. The results point to the fact that, as grain becomes moldy, the fat acidity increases, the activity of fungal lipase increases, and the wheat lipase activity decreases. This corroborates conclusions from respiratory experiments (10, 15, 16, 17) that the molds were primarily responsible for the increases in fat acidity which occur under conditions which permit deterioration of stored grain. It must be pointed out, however, that the assays were conducted in water extracts which may not represent the true natural conditions. For example, the inhibitor of fungal lipase in the germ or some other portion of the wheat kernel which is extractable by water may not be active in the relatively dry state in the seed, but may become active in the extract. If this is the case, the assay results may be misleading in regard to conditions in the natural state.

Only the inhibition of fungal lipase has been considered to this point. A reason for the obvious decreases in cereal lipase activity is also necessary. Similar observations were reported by Ramakrishnan and Banerjee (18) in which the lipase activities of castor bean and ground

nut (peanut) meals decreased as molds developed on them. These effects may be due to proteolytic activity of the mold which destroys the lipase protein as well as other essential proteins in the seed or meal. In this connection, Thomas (27) has noted that a toxin may be secreted by certain species of the *Aspergilli* which destroys the germinating power of the wheat seed. This toxin may be a proteolytic enzyme or a powerful enzyme inhibitor which is effective in inhibiting the cereal lipase as well as other enzymes necessary for germination.

Acknowledgments

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Literature Cited

1. Adams, D. H., and Thompson, R. H. S. The selective inhibition of cholinesterases. Biochem. J. **42:** 170–175 (1948).
2. American Association of Cereal Chemists. Cereal laboratory methods (5th ed.), p. 7. The Association: St. Paul, Minnesota (1947).
3. Bradshaw, R. C. A. Lipolytic enzymes, with special reference to the baking industry. Food Sci. Abstracts **24:** 501–510 (1952).
4. Christensen, C. M. The quantitative determination of molds in flour. Cereal Chem. **23:** 322–329 (1946).
5. Dirks, B. M. The properties of some fungal lipases and their role in grain deterioration. Ph.D. Thesis, Department of Agricultural Biochemistry, University of Minnesota, St. Paul, 1953.
6. Fiore, J. V., and Nord, F. F. On the mechanism of enzyme action. XLII. Isolation and some properties of the lipase from *Fusarium lini* Bolley. Arch. Biochem. **26:** 382–400 (1950).
7. Fodor, P. J., and Chari, A. The ester-hydrolysing enzyme systems of *Aspergillus niger* and of *Penicillium roqueforti*. Enzymologia **13:** 258–267 (1948).
8. Geddes, W. F. The role of enzymes in milling and baking technology. Food Technol. **4:** 441–446 (1950).
9. Goodman, J. J., and Christensen, C. M. Grain storage studies. XI. Lipolytic activity of fungi isolated from stored corn. Cereal Chem. **29:** 299–308 (1952).
10. Hummel, B. C. W., Cuendet, L. S., Christensen, C. M., and Geddes, W. F. Grain storage studies. XIII. Comparative changes in respiration, viability, and chemical composition of mold-free and mold-contaminated wheat upon storage. Cereal Chem. **31:** 143–150 (1954).
11. Kirsh, D. Factors influencing the activity of fungus lipase. J. Biol. Chem. **108:** 421–430 (1935).
12. Kleinzeller, A., and Trim, A. R. Use of a mixed indicator in the titration of fatty acids. Analyst **69:** 241 (1944).
13. Lineweaver, H., and Burk, D. The determination of enzyme dissociation constants. J. Am. Chem. Soc. **56:** 658–666 (1934).
14. Milner, M. Biological factors in damp grain deterioration. Food Technol. **5:** 25–30 (1951).
15. Milner, M., Christensen, C. M., and Geddes, W. F. Grain storage studies. V. Chemical and microbiological studies on "sick" wheat. Cereal Chem. **24:** 23–38 (1947).
16. Milner, M., Christensen, C. M., and Geddes, W. F. Grain storage studies. VI. Wheat respiration in relation to moisture content, mold growth, chemical deterioration, and heating. Cereal Chem. **24:** 182–199 (1947).

17. MILNER, M., and GEDDES, W. F. Grain storage studies. III. The relation between moisture content, mold growth, and respiration of soybeans. Cereal Chem. **23:** 225–247 (1946).
18. RAMAKRISHNAN, C. V., and BANERJEE, B. N. Destruction of the lipase in the oil seeds as the molds grow on them. J. Indian Chem. Soc. **28:** 591–594 (1951).
19. RAMAKRISHNAN, C. V., and BANERJEE, B. N. Studies on lipolytic molds. Comparative study of lipases obtained from molds grown on oil seeds. J. Am. Oil Chemists Soc. **29:** 596–601 (1952).
20. SHIPE, W. F., JR. A study of the relative specificity of lipases produced by *Penicillium roqueforti* and *Aspergillus niger*. Arch. Biochem. **30:** 165–179 (1951).
21. SINGER, T. P. On the mechanism of enzyme inhibition by sulfhydryl reagents. J. Biol. Chem. **174:** 11–21 (1948).
22. SINGER, T. P., and HOFSTEE, B. H. J. Studies on wheat germ lipase. I. Methods of estimation, purification, and general properties of the enzyme. Arch. Biochem. **18:** 229–243 (1948).
23. SINGER, T. P., and HOFSTEE, B. H. J. Studies on wheat germ lipase. II. Kinetics. Arch. Biochem. **18:** 245–259 (1948).
24. SULLIVAN, BETTY. Esterases in relation to milling and baking. *In* Enzymes and their role in wheat technology, ed. by J. A. Anderson, pp. 153–174. Interscience: New York (1946).
25. THIBODEAU, R., and MACY, H. Growth and enzyme activity of *Penicillium roqueforti*. Minn. Agr. Expt. Sta. Tech. Bull. 152 (June 1942).
26. THOM, C., and RAPER, K. B. A manual of the *Aspergilli*. Williams and Wilkins: Baltimore (1945).
27. THOMAS, R. C. The role of certain fungi in the "sick wheat" problem. Ohio Agr. Expt. Sta. Bimo. Bull. **22** (185): 43–45 (March-April 1937).
28. UMBREIT, W. W., BURRIS, R. H., and STAUFFER, J. F. Manometric techniques and related methods for the study of tissue metabolism (2nd ed.). Burgess: Minneapolis (1949).
29. WEBB, E. C. The action of alkyl fluorophosphonates on esterases and other enzymes. Biochem. J. **42:** 96–98 (1948).

ENZYMES THAT DEGRADE BARLEY GUMS. III. STUDIES OF BETA-POLYGLUCOSIDASES OF GREEN MALT[1]

E. J. Bass and W. O. S. Meredith

ABSTRACT

Products of the reaction between enzyme preparations from green malt and gum preparations from barley treated to inactivate enzymes were examined by descending paper chromatography. The two substrates used in this investigation were a barley gum containing glucose, arabinose, xylose, and a trace of galactose residues, and a beta-polyglucoside containing only glucose residues. Enzymic hydrolysis of the barley gum yielded an oligopentosan, oligoglucosides, a disaccharide (maltose or cellobiose), galactose, glucose, arabinose, and xylose. Enzymic hydrolysis of the beta-polyglucoside yielded oligoglucosides, cellobiose, glucose, and an unidentified disaccharide (possibly laminaribiose). The order of appearance of reaction products from beta-polyglucoside supports the postulated existence of an endo-beta-polyglucosidase and an exo-beta-polyglucosidase in green malt. It is suggested that the endo- and exo-enzymes are relatively nonspecific beta-polyglycosidases. A possible mechanism of cytolysis (cell wall degradation) in germinating barley is postulated.

Because barley gums are now generally believed to be structurally related to endosperm cell wall material, the gum-degrading, or cell wall decomposing, enzymes of barley and green malt have been the object of intensive research in recent years. In 1950, Preece and Ashworth (10), using barley gum preparations as substrates and reducing power as a measure of enzyme activity, suggested the existence of two cell wall degrading enzyme systems in germinating barley which they termed "cytoclastic" and "cytolytic" to differentiate between a disaggregating and a more profound hydrolytic action. Later, Sandegren and Enebo (14), using a commercial "ethylhydroxyethyl cellulose" preparation as substrate, presented a viscosimetric method of measuring the "cellulase" activity of barley and green malt. Simultaneously, Bass, Meredith, and Anderson (2) described a viscosimetric method for measuring the activity on barley gums of enzymes from a bacterial preparation and from green malt. Their results indicated that viscosity reduction was due to the action of an endo-glycosidase system present in both enzyme sources.

In 1953, Enebo, Sandegren, and Ljungdahl (5), continuing their investigation of the action of cell wall decomposing enzymes on their artificial substrate, postulated the existence of two cellulases in green malt – a beta-polyglycosidase and a transglycosidase. At the same time,

[1] Manuscript received April 6, 1955. Paper No. 148 of the Grain Research Laboratory, Board of Grain Commissioners for Canada, Winnipeg, and No. 327 of the Associate Committee on Grain Research (Canada). This paper is based on a Ph.D. thesis submitted by E. J. Bass to the University of Manitoba, Winnipeg, in September, 1954.

Bass, Meredith, and Anderson (3), using paper chromatography to identify the hydrolysis products liberated by green malt enzymes from barley gums, confirmed the existence of a beta-polyglycosidase system in green malt which, they suggested, consisted of an endo-beta-glucosanase and an endo-pentosanase. They further postulated the action of an exo-beta-glucosanase that liberates cellobiose from ends of beta-glucosan chains, and demonstrated the existence of cellobiase in green malt, thus accounting for the appearance of glucose among enzyme hydrolysis products. These views have apparently been accepted by Preece, Aitken, and Dick (13), who now also use the terminology "endo-beta-glucosanase" and "exo-beta-glucosanase."

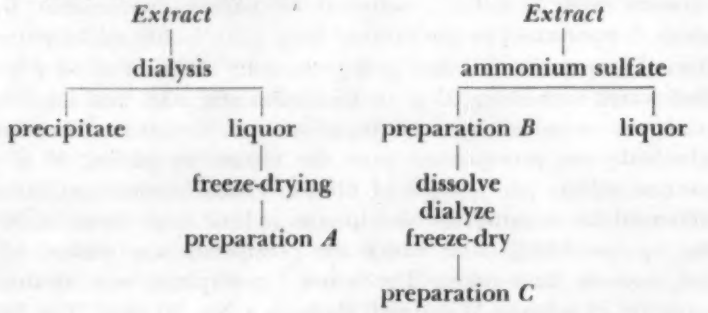
The present investigation is confined to enzymes of green malt, because enzyme concentrations are higher in green malt than in ungerminated barley. The enzymic reaction products of two barley gum preparations have been examined by descending paper chromatography, and additional evidence for the existence of an exo-beta-glucosanase in green malt has been obtained. The terminology has been altered somewhat to conform with polysaccharide nomenclature, "polyglucoside" being preferred to "glucosan."

Materials

Two substrates were used in this investigation: a barley gum previously referred to as "papain gum" (3), and a beta-polyglucoside similar to the beta-glucosan of Preece and Mackenzie (11). The barley gum contained glucose, arabinose, and xylose residues in the molecular ratio of 100:9:14, and also contained a trace of galactose. The beta-polyglucoside was prepared from barley grist refluxed with boiling 85% ethyl alcohol (sp. gr. 0.850) for 30 minutes to inactivate enzymes. The grist was centrifuged, air-dried, then mashed for 2 hours at room temperature with a 0.025% solution of papain (Nutritional Biochemicals Corporation) in the ratio of 50 g. grist to 400 ml. of papain solution. The papain was then precipitated by addition of 50 ml. of distilled water containing 27 g. of trichloroacetic acid, and insoluble material was removed by centrifugation and filtration. The beta-polyglucoside was precipitated from the filtrate by adding 30 g. of ammonium sulfate per 100 ml. of filtrate. Excess ammonium sulfate was removed by washing the precipitate at least four times in 50% alcohol (sp. gr. 0.934), after which the precipitate was washed with alcohol, acetone, and ether. The washed precipitate was air-dried, then ground in a micro Wiley mill through a No. 30 sieve. The final yield of material was about 1% of the grist (as is), and the relative viscosity at 30°C. of a 1% aqueous solution (as is) was about 100. This

product yielded only glucose on acid hydrolysis, gave no characteristic color with iodine in aqueous solution, and had a negative optical rotation. Hence this substrate is presumably a beta-linked polyglucoside, analogous to the methylated 1:4-beta-polyglucoside isolated from methylated barley gum by Gilles, Meredith, and Smith (8). However, there is now reason to believe (13) that glucose residues in the beta-polyglucoside may be linked by both 1:4-beta- and 1:3-beta-glucosidic linkages in approximately equal proportions. Preece and Mackenzie (11) consider the beta-polyglucoside to be the component mainly responsible for the characteristic properties of barley gum.

Enzyme sources were three preparations from extracts of green malt, malted by the Barley Improvement Institute, Winnipeg, from Montcalm barley grown at the University of Manitoba in 1952. The first preparation was obtained by dialyzing an extract of green malt for 24 hours against tap water, discarding the precipitate, and freeze-drying the liquor. The second was obtained by precipitation with ammonium sulfate in the ratio of 64 g. ammonium sulfate per 100 ml. of extract. The precipitate was collected by suction filtration through Celite, and the mixture of Celite and precipitate was dried *in vacuo* over phosphorus pentoxide. Clear, concentrated solutions of this material could be obtained by mixing the crude preparation and water for 30 minutes in a Burrell Wrist-Action Shaker, followed by suction filtration through Celite. The third preparation was obtained by dialyzing and freeze-drying the clear solution of the second preparation. Recoveries of viscosity-reducing activity, as determined by the procedure reported in previous papers (2, 3), were about 40% for the freeze-dried dialyzed extract, preparation *A*, and over 90% for the other two preparations. The three products are illustrated in the accompanying flow diagram.



Methods

Two procedures, both involving chromatography of enzymic hy-

drolysates, were used to detect enzyme-substrate reaction products. The first was similar to the method described in a previous paper (3), using barley gum as substrate. This procedure gave highly colored hydrolysates which produced colored spots at the base line of chromatograms even before spraying. These spots obscured the presence of oligosaccharides of relatively high molecular weight, occurring at or near the base line. In addition, chromatograms were often streaky and difficult to interpret, and enzyme blanks contained small amounts of the same carbohydrate components present in reaction mixtures. These difficulties were overcome by reducing the enzyme concentration in reaction mixtures, and by freeze-drying the solutions prior to chromatography.

The modified procedure for preparing and analyzing enzymic hydrolysis products was as follows. Reaction mixtures contained 10 ml. of 1% beta-polyglucoside (as is), 1 ml. of enzyme solution (containing the equivalent of 0.16 g. green malt), and 1 drop of toluene to inhibit bacteria. Blanks contained water instead of enzyme or substrate. Reaction mixtures and blanks were incubated at room temperature for various periods of time from 1 hour to 78 hours, during which time there was no visible evidence of bacterial growth. Enzymes were inactivated by heating for 20 minutes in a boiling-water bath, and the heated mixtures were then cooled and freeze-dried. Aliquots of 0.02 g. from reaction mixtures and substrate blanks, and of 0.002 g. from enzyme blanks were weighed accurately and dissolved in 0.2 ml. of distilled water. The proportionate weights of substrate and enzyme in blanks were so calculated as to be somewhat greater than their proportionate weights in reaction mixtures. Six microliters of each solution were then chromatographed for 30 hours at room temperature, using ethyl acetate/pyridine/water (5:2:5) as solvent. Aniline phosphate (4) was the spray reagent. Hydrolysates from reaction mixtures incubated for 6 hours or less, and from all substrate blanks, required larger amounts of water (0.4 and 0.6 ml. respectively), hence proportionately larger aliquots of these solutions were used for chromatography. All chromatograms were scanned with ultraviolet light to detect trace amounts of sugars.

Results

In a previous study (3) an enzyme preparation, obtained from green malt extract by precipitation with ammonium sulfate, liberated from barley gum one oligopentosan, at least one oligoglucoside, glucose, and a trace of galactose. The present investigation confirmed and expanded these findings. All three enzyme preparations yielded

the same products from barley gum, namely: an oligopentosan, one or more oligoglucosides, a trace of either maltose or cellobiose, a trace of galactose, and appreciable amounts of glucose, arabinose, and xylose. The enzyme solution, carried as a blank, produced the same products in much smaller amounts; these are apparently derived from the malt gums which are high in pentosan content (9). Preece *et al.* (13) have also noted that enzyme extracts are contaminated with gums and produce various sugars on autolysis.

The large number of products, and particularly the release of arabinose and xylose, are of considerable interest. However, the gum appears to be too complex a substrate for present studies of the enzymes. Accordingly, the enzymatic hydrolysis products of the beta-polyglucoside were examined next, and with more fruitful results. This compound represents about 75% of the barley gum obtained by papain treatment and alcohol precipitation, and contains only glucose, though in both 1:3 and 1:4 linkages.

Figure 1 shows the development of reaction products with time when the beta-polyglucoside was incubated with a dilute solution of enzyme preparation *B*. Enzyme and substrate blanks, not shown in Fig. 1, yielded no detectable products. A trace of glucose appeared after a 1-hour reaction period (column 1), and the amount of glucose, as judged by the intensity of the spots, increased continuously with increasing reaction time (columns 2 to 10). A trace of cellobiose appeared after 30 hours (column 6), and its concentration remained essentially unchanged for the balance of the reaction period. A trace of an unidentified disaccharide (possibly laminaribiose) appeared just below cellobiose after 30 hours, and it too remained essentially constant in amount for the balance of the reaction period. The first trace of an oligoglucoside appeared after 1 hour and gradually increased in amount up to 6 hours. As this oligosaccharide occurred at the base line, it is probably an oligoglucoside of relatively high molecular weight. After 24 hours (column 5), a second oligoglucoside appeared below the first, and two more oligoglucosides appeared beneath the second after 30 hours. The latter three oligoglucosides are presumably oligosaccharides of low molecular weight. The intensities of oligoglucoside spots appeared to decrease after 30 hours (column 6), and only traces of all four oligosaccharides were present after 78 hours. This suggests that the enzymatic degradation of beta-polyglucoside to oligoglucosides is virtually complete, or in equilibrium with resynthesis, after about 30 hours, and that the degradation of oligoglucosides to sugars continues beyond this reaction time.

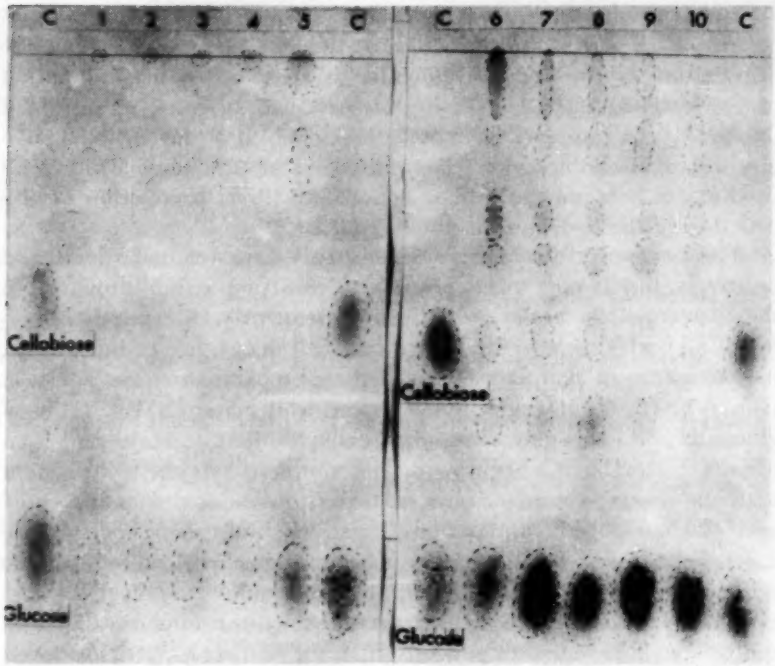


Fig. 1. Enzymatic hydrolysis products of beta-polyglucoside after various reaction times: 1, 1 hour; 2, 2 hours; 3, 4 hours; 4, 6 hours; 5, 24 hours; 6, 30 hours; 7, 48 hours; 8, 54 hours; 9, 72 hours; 10, 78 hours.

Discussion

There are three possible routes for the enzymatic degradation of the beta-polyglucoside. First, the substrate may be degraded by random glycosidic cleavages, requiring the action of only one enzyme. This mechanism would explain both the rapid decrease in solution viscosity and the simultaneous appearance of glucose and the high-molecular-weight oligoglucoside, in early stages of the reaction. However, if glycosidic linkages are ruptured at random, oligoglucosides of relatively low molecular weight also should be liberated early in the reaction. As these products were not detected during the first 6 hours, this mechanism appears to be unlikely. Second, the substrate may be progressively degraded by internal glycosidic cleavages only. This action explains both the rapid decrease in solution viscosity and the liberation of oligoglucosides of gradually decreasing molecular weight, but does not account for the appearance of glucose in early stages of the reaction. Finally, the beta-polyglucoside may be degraded by terminal glycosidic cleavages only. This action would have little immedi-

ate effect on solution viscosity, but would account for the release of glucose in initial stages of the reaction. It thus appears that the complete degradation of the beta-polyglucoside is achieved by a combination of the last two actions involving at least two enzymes, as suggested previously (3): an endo-beta-polyglucosidase and an exo-beta-polyglucosidase. Cellobiose and laminaribiose are the most likely final products of beta-polyglucosidase action, but direct production of glucose during the hydrolysis is not excluded.

The beta-polyglucosidases of green malt have yet to be identified positively, but it now seems possible to construct a simple picture of the enzymolysis of barley gums and, consequently, of cytolysis in germinating barley. Due to the known specificities of other carbohydrases, it is tempting to postulate the existence of separate enzymes for each type of glycosidic linkage present in cell wall materials, e.g. 1:4-beta-glucosidic, 1:3-beta-glucosidic, 1:4-beta-xylosidic (8). However, it is usually preferable to begin with the simplest hypothesis consistent with the observed results. Thus, it may be postulated that the endo- and exo-beta-polyglucosidases are relatively nonspecific polyglycosidases, both of which can degrade materials detached from endosperm cell walls (such as barley gums). Only the endo-beta-polyglycosidase can, however, attack the cell wall itself, since the impermeability of the cell wall to enzymes suggests that the molecules are so closely associated as to expose relatively few free chain ends to exo-action. Thus the endo-beta-polyglucosidase may well be the chief cytolytic, or cell wall degrading, enzyme. The first products of cytolysis are therefore high-molecular-weight polysaccharides, which, by competing with the intact cell wall for the endo-enzyme, inhibit further cytolysis. The removal of these inhibitors of cytolysis may then be the chief function of the exo-beta-polyglycosidase.

It would appear, then, that Sandegren's or Preece's viscosimetric cellulase determinations (12, 14) or our determination of viscosity-reducing activity (2, 3) might provide an additional test for malting quality. The apparent failure of Sandegren's barley cellulase determination in the recent European trials (6, p. 70; 7, p. 88) does not alter this conclusion, as Thunaeus and Sandegren have observed (15) that cellulase activity in barley is influenced by the stage of maturation of the barley. Hence ungerminated barley may be too variable for a test material. Possibly barley germinated to a given stage, such as green malt, may be more satisfactory.

A preliminary investigation (1, p. 112) of the viscosity-reducing activities of green malts from nine Canadian barley varieties grown at a single station has shown that this approach is promising. Results

were reproducible, large varietal differences (almost 100%) were observed, and activity appeared to be related to malting quality. A larger scale study is now under way, and a potential method for estimating exo-beta-polyglycosidase activity is being investigated so that varietal differences in exo-activity can be studied simultaneously with varietal differences in endo-beta-polyglycosidase activity.

Literature Cited

1. Bass, E. J. Cytolytic enzymes in germinating barley. Thesis, University of Manitoba (1954)
2. Bass, E. J., Meredith, W. O. S., and Anderson, J. A. Enzymes that degrade barley gums. I. Isolation from a bacterial source. Cereal Chem. **29:** 262–272 (1952).
3. Bass, E. J., Meredith, W. O. S., and Anderson, J. A. Enzymes that degrade barley gums. II. Preparations from barley and green malt. Cereal Chem. **30:** 313–322 (1953).
4. Bryson, J. L., and Mitchell, T. J. Improved spraying reagents for the detection of sugars on paper chromatograms. Nature **167:** 864 (1951).
5. Enebo, L., Sandegren, E., and Ljungdahl, L. Cell wall decomposing enzymes of barley and malt. II. Cellulase increase during germination and influence of sugars on cellulase activity. J. Inst. Brewing **59:** 205–211 (1953).
6. European Brewery Convention. Report of the Barley Committee. Rotterdam. (1951).
7. European Brewery Convention. Report of the Barley Committee. Rotterdam. (1952).
8. Gilles, K. A., Meredith, W. O. S., and Smith, F. The carbohydrates of the *Gramineae*. The constitution of the water-soluble polysaccharides of the grain of barley (*Hordeum vulgare*). Cereal Chem. **29:** 314–316 (1952).
9. Meredith, W. O. S., and Anderson, J. A. Comparison between malt and barley gums isolated by various procedures. Cereal Chem. **32:** 183–191 (1955).
10. Preece, I. A., and Ashworth, A. S. Cytolysis in germinating barley. II. Preliminary study of enzyme relationships. J. Inst. Brewing **56:** 40–47 (1950).
11. Preece, I. A., and Mackenzie, K. G. Non-starchy polysaccharides of cereal grains. I. Fractionation of the barley gums. J. Inst. Brewing **58:** 353–362 (1952).
12. Preece, I. A., and Aitken, R. A. Non-starchy polysaccharides of cereal grains. IV. Cellulase activity and autolysis relationships of some malting barleys. J. Inst. Brewing **59:** 453–461 (1953).
13. Preece, I. A., Aitken, R. A., and [in part] Dick, J. A. Non-starchy polysaccharides of cereal grains. VI. Preliminary study of the enzymolysis of barley β-glucosan. J. Inst. Brewing **60:** 497–507 (1954).
14. Sandegren, E., and Enebo, L. Cell wall decomposing enzymes of barley and malt. I. Determination and stability investigations. J. Inst. Brewing **58:** 198–203 (1952).
15. Thunaeus, H., and Sandegren, E. Barley trials 1953 (Sweden). Report of the Barley Committee, Trials 1953: 76–82. European Brewery Convention. Rotterdam, 1955.

A STUDY OF THE LACTIC ACID FERMENTATION IN COMMERCIAL CORN STEEPING[1]

STANLEY A. WATSON, YOSHIRO HIRATA, AND CARL B. WILLIAMS

ABSTRACT

Fresh corn steepwater incubated at 48°C. in the presence of excess dextrose forms lactic acid in an amount which is related to the population of lactic acid bacteria. Application of this principle as a method of determining relative bacterial activity (RBA) has revealed that bacterial inoculation of steepwater may come either from wet milling process water or from raw grain, but process water has a relatively greater population of lactic acid bacteria.

In the sequence of events in commercial steeps containing corn and process water held at 48°–50°C., reducing sugars accumulated in the water during the first 16–24 hours. Concentration of sugars dropped rapidly when the population of lactic acid bacteria, as indicated by high RBA values, became well established. Disappearance of sugar was accompanied by an increase in lactic acid, a decrease in pH, and an increase in free amino acids. Extraction of total and phytin phosphorus was also affected by the lactic acid production.

Interruption in battery steeping caused by week-end shutdown resulted in gradual dying out of bacteria due to exhaustion of substrate. Following resumption of milling and steeping operations, bacterial activity in steepwater dropped to zero. Simultaneously, reducing sugar concentration and pH increased, and lactic acid concentration decreased.

Manufacture of starch from corn involves a preparative steeping of the corn for 40 to 50 hours. Steeping temperatures in the range of 49°–53°C. (120°–127°F.) have been found to result in optimum starch recovery and quality, maximum germ separation, and the optimum rate of steepwater evaporation. In this temperature range, maximum amounts of lactic acid are formed by the action of various types of homofermentative lactic acid bacteria from reducing sugars, which are leached from the corn. These bacteria are able to grow at higher temperatures and tolerate higher acid concentrations than most other microorganisms. They also require very little oxygen and tolerate some sulfur dioxide (3, 8). Lactic acid is necessary in commercial steeping practice because it is an essential part of the buffer system that keeps the steepwater near pH 4. At this pH undesirable microorganisms are inhibited, and the steeped corn has better milling properties. It is apparent, therefore, that operation of a steep battery is closely related to the conditions required for optimum growth of the indigenous species of lactic acid bacteria.

The present work was undertaken to learn more about the prac-

[1] Manuscript received May 25, 1954. Presented at the 39th Annual Meeting at Denver, Colorado, May, 1954. Contribution of the George M. Moffett Research Laboratories, Corn Products Refining Company, Argo, Illinois.

tical requirements of the fermentation phase of the steeping operation in order to provide information which will aid in its control. The relative bacterial activity (RBA) method described in a previous publication (11) was used in the present investigation with slight modification. Classical bacteriological methods, although generally quite precise, are tedious and time-consuming, and require specialized technic. Furthermore, the results may be difficult to interpret for guiding steeping practice. The RBA method is simple and direct. It gives useful information because it provides a direct estimation of lactic acid fermentation. Application of this method to a variety of steeping problems is described, and results are interpreted in relation to more commonly used methods of steepwater characterization.

Materials and Methods

Overflow water from gluten settlers was obtained at the Argo, Illinois, plant of Corn Products Refining Company. The seed grade corn (Sample A) was grown near Clinton, Illinois, in 1949. It has been kept in cold storage. All other corn samples were obtained shortly before use from commercial channels through the Chicago Board of Trade.

Relative Bacterial Activity. Although this method was briefly outlined in a previous publication (11), a different incubation temperature is now preferred. The method is here given in complete detail.

Reagents: (1) Dextrose solution, 50% by weight.

(2) Steepwater nutrient solution containing 22% solids. Diluted from commercial concentrated steepwater (55% solids).

(3) Sodium hydroxide solution, 10% by weight.

(4) Standard sodium hydroxide solution, 0.1 *N*.

Procedure: The sample of steepwater to be tested was thoroughly mixed but was not filtered. A 20-ml. aliquot of the steepwater and 2 ml. each of the dextrose and steepwater nutrient solutions were placed in a 100-ml. beaker. With stirring, 10% sodium hydroxide solution was added slowly to about pH 4.8, then 0.1 *N* sodium hydroxide was cautiously added to exactly pH 5.0. The sample was then diluted to 50 ml. in a test tube (22 × 150 mm.) and mixed. The tube was incubated 4 hours at 48°–48.5°C. (118°–119°F.) with no agitation. The tube was then cooled, and the contents were titrated with 0.1 *N* standard sodium hydroxide solution to exactly pH 5.0.

Relative bacterial activity (RBA) is defined as the milligrams of lactic acid formed by the bacteria in 100 ml. of original steepwater in the presence of an excess of dextrose under the specified incubation conditions: $\text{RBA} = \text{ml. } 0.1\ N\ \text{NaOH} \times 45$

Nitrogen. Total nitrogen was determined by the A.O.A.C. official Gunning method (1). A formol titration procedure (9) was adapted for use in determining free amino acids in steepwater. A 10-ml. sample of steepwater was adjusted to exactly pH 8.0 (electrometric) with sodium hydroxide solution. With stirring, 25 ml. of 40% formaldehyde solution (previously adjusted to pH 8.0) then were added to the steepwater. The mixture was titrated with standard 0.1 *N* sodium hydroxide solution to pH 7.5–7.8 and stirred 3 minutes; then the titration was completed to exactly pH 8.0. Free amino nitrogen was assumed equivalent (mole for mole) to the sodium hydroxide required.

Phosphorus. Samples were digested with sulfuric acid, and total phosphorus was determined by the colorimetric procedure of Kitson and Mellon (7). Phytin was precipitated as the iron salt from hot acidified steepwater (4), and phosphorus was determined on the precipitate.

Reducing Sugars. Steepwater was treated with phosphotungstic acid to remove proteinaceous substances. An aliquot of the filtrate was analyzed for reducing sugars by the Schoorl copper reduction method (2). Results were expressed as dextrose.

Total Acidity. A 10-ml. sample of steepwater was titrated with 0.1 *N* sodium hydroxide to exactly pH 8.0. Results were expressed as lactic acid. Agreement with a more precise permanganate oxidation method (10) was satisfactory.

Sulfur Dioxide. Sulfur dioxide was determined by titration with standard iodine-potassium iodide solution adjusted to be equivalent to 0.001 g. sulfur dioxide per milliliter. Soluble starch was used as the indicator.

Dry Substance. Total dissolved solids in steepwater were determined from tables relating density readings in degrees Baumé to dry substance values in g. per 100 ml.[2]

Sources of Inoculation in Steeping

Raw grain entering the steeping system has long been considered to be the major source of lactic acid bacteria. However, several experiences suggested that the supernatant water from the gluten settlers might be of greater importance than the grain in the initiation of fermentation. This water is a by-product of the wet milling process. It is first introduced into the system as fresh water for washing starch (5) and is subsequently reused as a medium for milling and separating starch. The water recovered contains about 1% dissolved solids following concentration of gluten by settling or by centrifugation. It is

[2] Unpublished work: F. W. Fuchs, Corn Products Refining Company.

then commonly used as a steeping medium after the addition of 0.1 to 0.2% sulfur dioxide. Experiments were carried out to determine the relative importance of this water and of corn in initiation of fermentation in the steeps.

Six hundred grams of corn (d.b.) in a 2-quart jar were covered with 950 ml. overflow water from the gluten settlers to which had been added sulfur dioxide to a concentration of 0.095 g. per 100 ml. Four sets of comparisons were made in duplicate: 1) raw corn with raw overflow water, 2) raw corn with sterilized overflow water, 3) "sterilized" corn with raw overflow water, and 4) "sterilized" corn with sterilized overflow water.

The fresh overflow water was obtained from the gluten settlers on the same day it was to be used. Some of this water was sterilized by heating at 121°C. for 30 minutes in a steam autoclave. Surface sterilization of corn was attempted, with only partial success, by washing with a 0.05% solution of the wetting agent, Aersol OT (American

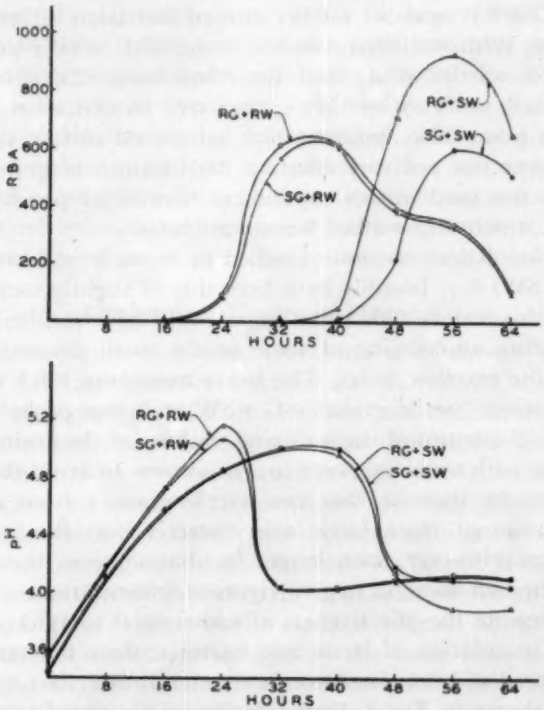


Fig. 1 Changes in the relative bacterial activity and pH in raw (RW) and sterile (SW) overflow water from the gluten settlers incubated at 49°C. (120°F.) with raw corn (RG) and "sterile" corn (SG).

Cyanamid Company). The grain was stirred vigorously by hand in the Aersol solution for 2 minutes, washed with water from a spray for 2 minutes, and then blotted on a sterile towel. Moisture content of the washed grain was determined and additional water was added to jars containing unwashed grain so that all jars would contain the same weight of water. The jars were covered and incubated at 49°C. (120°F.) in a constant-temperature water bath. They were mixed by inversion every 4 hours. Every 8 hours aliquots of water were removed for determination of pH, RBA, and sulfur dioxide.

The results, averaged and plotted against time in hours, are given in Fig. 1. Sulfur dioxide was not plotted because its concentration in all jars was close to 0.038 g. per 100 ml. after 8 hours and 0.030 g. per 100 ml. after 48 hours. The pH and RBA values closely followed an inverse relationship. The pH values rose to a peak of 5.0 to 5.2, declined sharply when RBA values started to increase, and then leveled off at close to pH 4 when RBA values had reached a maximum. All runs containing raw overflow water from the gluten settlers (RG + RW and SG + RW) showed initiation of fermentation at 16 hours. With sterilized overflow water (RG + SW) the onset of fermentation was delayed until the 32nd hour. "Sterilized" grain with sterilized water (SG + SW) gave active fermentation beginning at the 40th hour. This indicates that attempted surface sterilization with detergent was not very effective. Sterilization of grain by autoclaving was not used in this experiment because of possible changes in the corn which might affect fermentation.

The higher RBA maximum reached in the raw grain-sterile water run (RG + SW) may possibly have been due to slightly more dextrose present in this water. This extra sugar could have resulted from hydrolysis during autoclaving of some of the small amount of starch present in the overflow water. The lower maximum RBA reached in the sterile water-"sterile grain" (SG + SW) run was probably due to loss of a small amount of sugar during washing of the grain, although contact time with water was kept to a minimum to avoid this effect.

These results indicate that raw overflow water from the gluten settlers introduced more lactic acid bacteria into the system than did the corn. However, with longer incubation time, the raw grain furnished enough bacteria to give vigorous fermentation.

To determine the effectiveness of commercial samples of corn in furnishing inoculation of lactic acid bacteria, three different samples were incubated with sterilized overflow water as described above. RBA results are shown in Fig. 2. Fermentation was initiated soon after 32 hours in the steeps containing the commercial corn samples B and C.

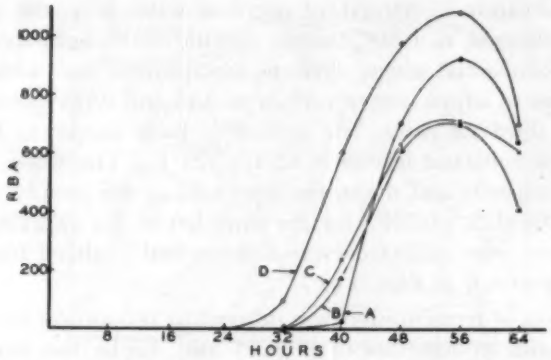


Fig. 2. Changes in relative bacterial activity in sterile overflow water from the gluten settlers incubated at 49°C. (120°F.) with raw corn from different sources. A, seed grade corn; B, C, and D, different lots of commercial corn of unknown histories.

In the steeps containing sample D, fermentation was initiated about 6 hours sooner. This indicates a greater natural bacterial population on corn sample D. All curves were similar in shape to the curve obtained with the seed grade corn A, although with this sample the fermentation was not initiated until almost the 40th hour. It is concluded that most lots of corn carry sufficient numbers of lactic acid bacteria to provide the needed fermentation in steeping. However, use of fresh overflow water from the gluten settlers will ensure earlier establishment of adequate fermentation. Overflow water from a well-regulated starch manufacturing system has been found to be fairly uniform from day to day in its ability to initiate fermentation.

Biochemical Events in Steeping

The exact sequence of events that occurs when corn is steeped in the presence of a lactic acid fermentation has never fully been studied. Such events are extremely difficult to follow in a commercial countercurrent battery because of mixing of water from different phases of the system. Besides, the unsteeped corn is immersed in water that has already reached its peak of fermentative activity. Therefore, the technic used in the laboratory in the previous section was applied to an experiment carried out in commercial size steeps. This involves the initial immersion of raw corn in raw overflow water from the gluten settlers. Thus changes in the medium effected by the corn can be distinguished from effects brought by the lactic acid bacteria. As will be seen, some effects of the fermentation on the corn also can be observed. It should be understood this method of steeping is the reverse of countercurrent steeping.

Approximately 15,000 gal. of overflow water from the gluten settlers was charged to 0.10% sulfur dioxide and was placed in each of three commercial steeps. Sodium metabisulfite was added to two of the steeps to adjust concentrations to 0.15 and 0.125%, respectively, while the third steep was left at 0.10%. Each steep was filled with 3,500 bu. of corn and heated to 52°C. (125°F.). The water was circulated continuously and the steeps were held in the temperature range of 50°–52°C. (122°–125°F.) for the duration of the experiment. Samples of water were collected every 6 hours and analyzed immediately. Results are shown in Figs. 3 to 7.

Initiation of fermentation was delayed in proportion to the initial sulfur dioxide concentrations (Figs. 3 and 4). In the steep having initially 0.15% sulfur dioxide, the minimum level reached was 0.05% sulfur dioxide in 30 hours, the time at which maximum RBA was reached. This is of interest because it had previously been thought that the lactic acid bacteria would not grow in a steep containing 0.05 to 0.06% sulfur dioxide. Killinger (6) pointed out that a small amount of fresh sulfur dioxide added to overflow water from gluten settlers was much more toxic than a larger amount of "old" sulfur dioxide, even though both were titratable with iodine. The sulfur dioxide concentration in the steep initially containing 0.10% sulfur dioxide (Fig. 3) showed a secondary rise at about 22 hours. This secondary rise is thought to be due to extraction of a sulfur dioxide compound formed within the corn kernel, because corn steeped in a lactic acid solution prior to contact with a sulfur dioxide solution does not show this rise.

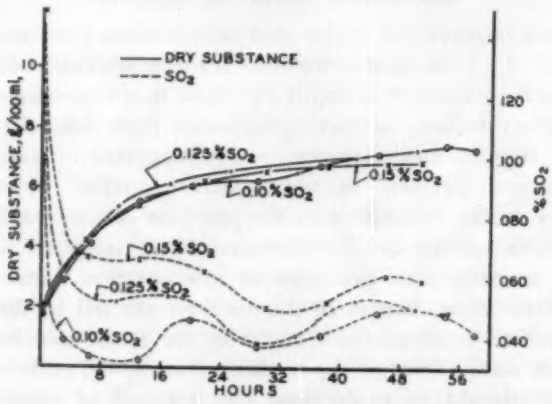


Fig. 3. Changes in dry substance and sulfur dioxide content in steeping medium at three initial sulfur dioxide levels during incubation with corn at 50°-52°C. (122°-125°F.).

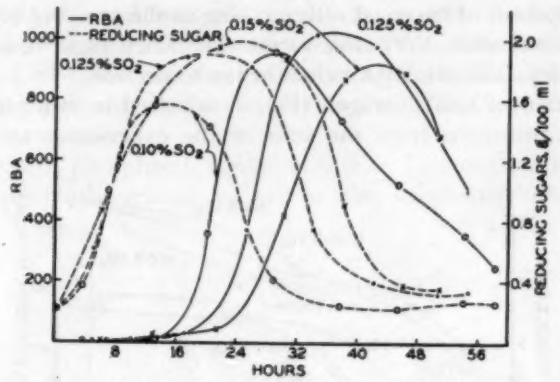


Fig. 4. Changes in reducing-sugar concentration and relative bacterial activity in steeping medium at three initial sulfur dioxide levels during incubation with corn at 50°-52°C. (122°-125°F.).

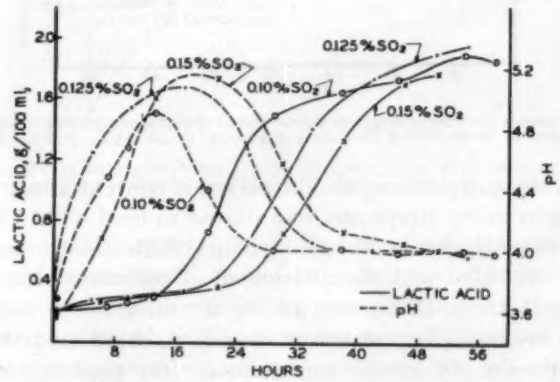


Fig. 5. Changes in pH and lactic acid concentration in steeping medium at three initial sulfur dioxide levels during incubation with corn at 50°-52°C. (122°-125°F.).

The rate of extraction of reducing sugars from the corn, shown in Fig. 4, was not affected by sulfur dioxide concentration. The amount of sugar which appeared in the steepwater, however, was affected by the sulfur dioxide level indirectly through the influence of sulfur dioxide on the time of initiation of fermentation. The maximum amount of sugar apparently had not been extracted from the corn in the 0.1% sulfur dioxide steep before fermentation started to consume it. A close relationship of the peaks and valleys in the curves for reducing sugars, pH, lactic acid, and RBA can be seen in Figs. 4 and 5. The reducing sugar and pH levels began to decline at exactly the same time the RBA and lactic acid levels started their abrupt rise. The RBA curves exhibit the lag, log, and decline phases so typical of

the development of bacterial cells growing in the presence of a limited amount of substrate. Very close to the time when sugar concentration started to level off, the RBA values began to decline.

Extraction of total nitrogen (Fig. 6) occurred in two phases. The rate was continuous from the start of the experiment to about 24

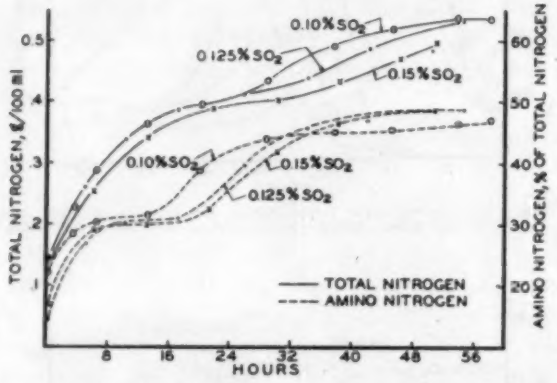


Fig. 6. Changes in total nitrogen and free amino nitrogen in steeping medium at three initial sulfur dioxide levels during incubation with corn at 50°-52°C. (122°-125°F.).

hours and was independent of sulfur dioxide concentration. Although total nitrogen in the steepwater had started to level off at 24 hours, it took a noticeable rise at about 30 hours. This change in nitrogen extraction coincided with the attainment of optimum lactic acid and minimum pH levels. Differences among the three steeps was probably due to an indirect effect of sulfur dioxide as it affected the time at which minimum pH levels were reached. One possible explanation for this secondary rise in total nitrogen may be the extraction of an acid-soluble form of nitrogen.

Amino nitrogen is plotted as a percentage of total nitrogen to show trends in the relative proportion of free amino acids to total nitrogen as possible evidence of active proteolysis. The curves in Fig. 6 show that amino nitrogen increased faster than total nitrogen between 14 and 34 hours. This phase coincided closely with the period of active bacterial growth shown in Fig. 4. The time placement of the increased amino nitrogen levels is related to the inhibition of bacterial growth as affected by initial sulfur dioxide levels. These observations suggest the presence of proteolytic enzymes of bacterial origin. Lactic acid bacteria are normally thought to be incapable of hydrolyzing peptide bonds. It may be that the particular strain of lactic acid bacteria present in steepwater has developed proteolytic enzymes or that one

or more nonlactic-acid bacterial types are present. Bruner (3) has examined actively fermenting steepwater and has differentiated four strains of *Lactobacillus* and three other cell types, including a Sarcina-like organism, probably *Pediococcus cerevisiae*, a *Bacillus*, and a Leuconostoc-like organism.

Extraction of phosphorus compounds (Fig. 7) showed a two-phase behavior apparently related to pH of the steepwater. Phosphorus

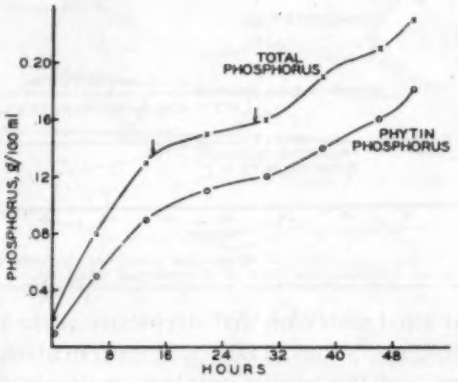


Fig. 7. Changes in total phosphorus and phytin phosphorus concentrations in steeping medium at three initial sulfur dioxide levels during incubation with corn at 50°-52°C. (122°-125°F.).

extraction was at first rapid, but in the period where sulfur dioxide and lactic acid levels were low and pH was high, the rate of phosphorus extraction leveled off. When enough lactic acid had been formed to cause the pH to drop, the extraction of phosphorus was resumed. The nonphytin phosphorus in steepwater is known to be about 50% orthophosphate. Steepwater does not contain phytase.

Effects of Interruptions in Battery Steeping Operations

Sequence of events in commercial steeping will not be precisely the same as has been shown in the above experiments, because steeps are normally operated in a countercurrent battery as a continuous operation whenever corn is being milled. During continuous steeping operations of a commercial countercurrent steeping battery, the bacterial population is normally maintained in equilibrium with the nutrient supply. The concentration of reducing sugars is thereby kept at a minimum level of 0.1 to 0.2%. However, manufacturing operations are generally suspended every week end for periods varying from 24 to 72 hours. When this happens, the flow of water through the steeps is stopped, and the steeps remain filled with corn and

water for the duration of the shutdown period. This imposes a new set of equilibrium conditions which produce changes in composition and bacterial activity of steepwater. In Fig. 8 are shown the changes

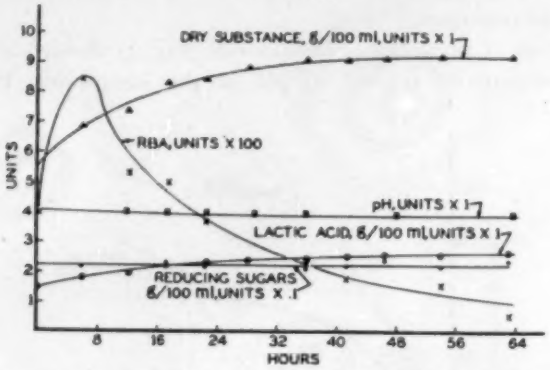


Fig. 8. Changes in the composition of steepwater remaining on corn at 50°-52°C. (122°-125°F.) during the hours following the start of a week-end shutdown.

in the steep last filled with corn and steepwater at the close of a week of normal milling operations. The water was circulated continuously within the steep and the temperature was maintained at 50°–52°C. (122°–125°F.) during the week-end.

The RBA level quickly rose to a peak in about 8 hours. After this it declined gradually to a very low level of activity beyond 60 hours, probably due to exhaustion of substrate. Although reducing sugar concentration in the steepwater declined slightly to a constant minimum value by the 12th hour, a continual increase in the concentration of lactic acid for about 40 hours showed that reducing sugars were continually leached from the corn and were fermented to lactic acid. Steepwater is not normally circulated during a shutdown, but the same changes have been found in quiescent steeps. Although the temperature of this steep was maintained by circulation of the steepwater through a heat exchanger, temperature increases of 1°–2°C. frequently have been recorded in the center of a quiescent steep during a 2-day shutdown. This temperature rise is believed to have resulted from energy released by the fermentation reaction.

The above results help to explain fluctuations often observed in bacterial activity and composition of steepwater after start-up of a countercurrent battery subsequent to a week-end shutdown. Figure 9 shows the changes observed during a typical week in the steepwater withdrawn from a commercial countercurrent battery. Results from two parallel batteries have been averaged. The prolonged leach-

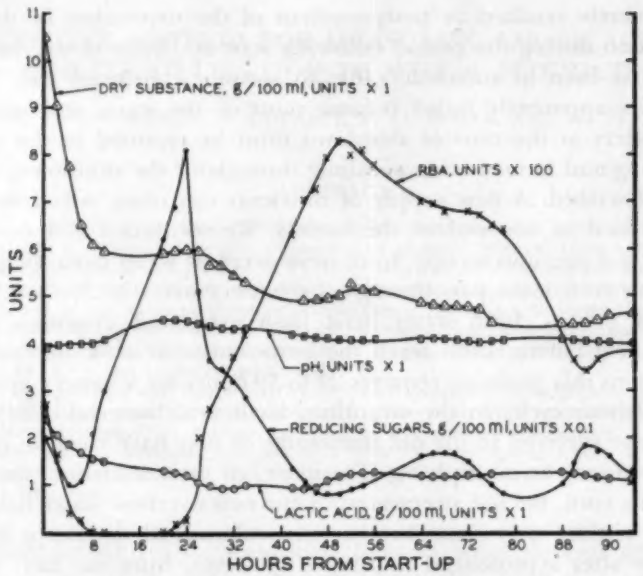


Fig. 9. Variation in composition of steepwater withdrawn from commercial countercurrent steep batteries during a week of normal operation following a 48-hour shutdown.

ing of the corn during the shutdown resulted in a high solids content in steepwater first withdrawn from the battery. The RBA level was at first relatively high, but dropped to zero by the 8th hour and remained at zero for about 12 hours. During this period reducing sugars were built up to a high concentration and lactic acid dropped to a low concentration, with a consequent increase in pH. At about the 20th hour from start-up the bacterial activity began to increase, reaching a peak of activity at about the 56th hour. Simultaneously, lactic acid concentration rose to a maximum level, and pH and reducing sugar concentration dropped to a minimum level. Small fluctuations after the middle of the week usually are not ascribed to shutdown effects.

The major lag in fermentation which occurred in the first 24 hours following start-up is attributed to the death of bacteria due to exhaustion of reducing sugars during the week-end shutdown. This loss in fermentation activity with consequent lower lactic acid concentration and higher pH is undesirable in the interests of uniform steeping. Attempts to eliminate or ameliorate these fluctuations in steepwater composition have not been successful.

Such methods as delaying the filling of steeps, inhibiting fermentation with sulfur dioxide, or sustaining fermentation by small additions of raw corn to the last steep throughout the shutdown period

have merely resulted in postponement of the depression in the fermentation during the period following start-up. None of the methods tried has been of sufficient value to warrant continued use. These remedies apparently failed because most of the water and grain in the battery at the time of shutdown must be retained in the steeps. Leaching and fermentation continue throughout the shutdown, as has been described. A new supply of nutrients can come only from raw corn added at one end of the battery. Reinoculation can come, as shown in a previous section, from fresh overflow water from the gluten settlers which must pass through the battery over the leached grain. In this process, fresh water must push exhausted shutdown water ahead of it before it can reach the fermentation zone. Under average conditions this probably requires 20 to 30 hours for a battery operated on a 40-hour cycle. In the meantime, lactic acid bacterial cells which may have survived in the old steepwater or may have come in on the new corn may be multiplying. Therefore, all three sources of bacterial cells, the corn, the old steepwater, or the new overflow water from the gluten settlers, may contribute to reinoculation of the steeps during start-up after a prolonged shutdown. However, from the facts which have been learned so far, the overflow water from the gluten settlers appears to be the major contributor of lactic acid bacteria.

Literature Cited

1. Association of Official Agricultural Chemists. Official Methods of Analysis (7th ed.). The Association: Washington, D. C. (1950).
2. Browne, C. A., and Zerban, F. W. Physical and chemical methods of sugar analysis (3rd ed.), p. 829. Wiley: New York (1941).
3. Bruner, R. L. Research progress reports. Corn Products Refining Company, Argo, Illinois (1951).
4. Earley, E. B. Determining phytin phosphorus. Stoichiometric relations of iron and phosphorus in ferric phytate. Anal. Chem. **16:** 389–391 (1944).
5. Kerr, R. W. Chemistry and industry of starch (2nd ed.). Academic Press: New York (1950).
6. Killinger, J. E. Factors affecting germicidal properties of sulfur dioxide in wet-starch-process waters. Ph.D. Thesis, Iowa State College, Ames, Iowa (1938).
7. Kitson, R. E., and Mellon, M. G. Colorimetric determination of phosphorus as molybdivanadophosphoric acid. Anal. Chem. **16:** 379–383 (1944).
8. Liggett, R. W., and Koffler, H. Corn steep liquor in microbiology. Bact. Rev. **12:** 297–311 (1948).
9. Northrop, J. H. A convenient method for the formol titration. J. Gen. Physiol. **9:** 767–769 (1926).
10. Smith, R. J. Determination of lactic acid in heavy corn steep liquor. Anal. Chem. **25:** 505–507 (1953).
11. Watson, S. A., Williams, C. B., and Wakely, R. D. Laboratory steeping procedures used in a wet milling research program. Cereal Chem. **28:** 105–118 (1951).

A SENSITIVE METHOD FOR MEASURING LIPASE ACTIVITY AND ITS APPLICATION TO WHEAT PRODUCTS[1]

W. W. Luchsinger, L. S. Cuendet, P. D. Boyer, and W. F. Geddes

ABSTRACT

A sensitive assay has been developed for measuring low levels of lipase activity. It is based upon the estimation of glycerol released from a monoolein emulsion at pH 7.4 and 30°C. After inactivation of the enzyme and destruction of the emulsion with trichloracetic acid and calcium chloride, the glycerol in the aqueous phase is determined by periodate cleavage, reaction of the resulting formaldehyde with chromotropic acid, and measurement of the absorbance of the colored complex at 570 mμ. Quantities of glycerol from 0.2 to 18.0 γ can be measured directly.

For assaying the lipase activity of wheat products, homogenates of the residue after successive extraction with petroleum ether and ammonium sulfate solution (95% saturation) were used as the enzyme source. Lipase values for the principal mill products from hard red spring wheat were in good relative agreement with limited available data by other methods in the literature.

Most procedures for measuring lipase activity depend upon the measurement of the acids released during the reaction (1, 12, 23, 25, 26). Manometric (17, 19, 22) and titrimetric methods (9, 10, 24, 28) have been employed to determine the lipase activity of cereals and cereal products, but none of the available methods appears to be sufficiently sensitive for accurate measurement of the low activities of highly refined wheat flours.

In an endeavor to develop a more sensitive assay for lipase, the possibility of quantitatively estimating the glycerol released upon hydrolysis was considered. Desnuelle and Naudet (3, 4) measured the glycerol released when pancreatic lipase acted on triglycerides by cleaving the glycerol with periodate and allowing the resulting formaldehyde to react with phenylhydrazine. A more sensitive and specific procedure for measuring formaldehyde was reported in 1937 by Eegriwe (6). The formaldehyde was reacted with chromotropic acid (1,8-dihydroxynaphthalene-3,6-disulfonic acid) in sulfuric acid to form a colored complex the quantity of which was determined by measuring the absorbance at 570 mμ. MacFayden (13) adapted the chromotropic

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acid method to measure formaldehyde in biological mixtures and later Lambert and Neish (11) combined the procedure with periodate oxidation to measure the glycerol produced in fermenting solutions.

This paper describes a procedure for determining lipase activity by measuring the glycerol released from a monoolein emulsion. The glycerol is separated from the substrate on the basis of its solubility in water and measured by determining the formaldehyde produced by periodate cleavage. Steapsin was used as the enzyme source in developing the method, which was then adapted to the determination of lipase activity in a series of wheat mill streams.

Development of the Method with Steapsin

Preparation of the Enzyme. Commercial steapsin (pancreatic lipase, Fisher Scientific Co., Pittsburgh, Pa.) was used since it was readily available and has been quite extensively studied (3, 8, 18, 21, 25). The enzyme was dissolved in cold 0.004 *M* ethylene-diamine-tetra-acetic acid (EDTA) in 0.004 *M* cysteine hydrochloride solution previously brought to pH 7.3 with sodium hydroxide to give a final enzyme concentration of 50 γ per ml.

Substrate. Ten grams of commercial monoolein (Glyco Products, Inc., Brooklyn, N.Y.), previously dissolved in ether and washed successively with sodium bicarbonate solution (2 g. salt + 98 g. water) and water, were added to a solution containing 0.1 g. of bile salts mixture (Difco Laboratories, Detroit, Michigan) consisting of 75% sodium taurocholate plus 25% accompanying bile acid salts and 89.9 ml. of distilled water, and whipped for 5 minutes in a Waring Blendor under nitrogen. The resulting emulsion was heated under reflux for 45 minutes in a covered boiling-water bath and stored at 3°C. This stock solution was diluted with 1.5 volumes of distilled water before use.

Buffer. 0.1 *M* potassium phosphate buffer, pH 7.4.

Inactivating Reagent. Five grams of calcium chloride dihydrate and 10 g. of trichloracetic acid were dissolved in 85 ml. of distilled water. An amount of this reagent equal to one-tenth of the reaction volume was added to inactivate the enzyme and to break the emulsion.

Glycerol Assay Reagents. 1. Periodic acid. A solution 0.1 *M* in periodic acid and 2.4 *N* in sulfuric acid.

2. Sodium arsenite, 1.0 *M*.

3. Chromotropic acid reagent. One gram of chromotropic acid (Matheson Chemical Co., East Rutford, N. J.) was dissolved in 100 ml. distilled water and filtered; the volume was adjusted to 500 ml. by the addition of a mixture of sulfuric acid (2 volumes) and water (1

volume). The solution was stored in a brown glass-stoppered bottle and prepared fresh every 2 weeks.

Method. A test-tube containing 1 ml. of monoolein substrate, 1 ml. of phosphate buffer, and 1 ml. of distilled water was placed in a water bath at 30°C. for 10 to 15 minutes. After temperature equilibration, 1 ml. of enzyme solution at 30°C. was added and the mixture incubated for 30 minutes. The final reaction mixture was 0.025 *M* in phosphate, 0.001 *M* in EDTA, 0.001 *M* in cysteine, approximately 1% in monoolein, and contained 12.5 γ of steapsin per ml. The initial pH was 7.40 and dropped to from 7.30 to 7.35 during the reaction. An appropriate control was prepared by adding the inactivating reagent and the enzyme at the end of the 30-minute reaction period.[2]

Following incubation, 0.4 ml. of inactivating reagent was added, and the mixture was shaken thoroughly and centrifuged 10 minutes in an International No. 2 centrifuge (head No. 240) at approximately 3000 r.p.m. A 1-ml. aliquot of the aqueous phase was transferred to one of three matched colorimeter tubes (the other two being a zero time blank and a water blank); 0.1 ml. of periodic acid-sulfuric acid solution was added, and exactly 5 minutes later, 0.1 ml. of the sodium arsenite solution; after which the mixture was shaken and allowed to stand for 5–10 minutes. Ten milliliters of chromotropic acid-sulfuric acid reagent were then added and the solution was heated in a covered boiling-water bath for 30 minutes. The solution was cooled in a water bath to 23°–27°C. and the absorbance read at 570 mμ in a Coleman Junior Spectrophotometer against an appropriate blank in which water replaced the aliquot from the enzyme reaction mixture.

Results. To establish the procedure, a glycerol standard curve, the recovery of glycerol from the reaction mixture, the relationship between substrate concentration and activity, and the relationship of enzyme concentration to glycerol released were determined. A few tests were also conducted to evaluate the reproducibility of the method.

For the glycerol standard curve, known amounts of C. P. glycerol were placed in matched tubes, the inactivating reagent was added and cleaved with periodic acid, and the color was developed according to the procedure previously described. The results of four separate trials, presented in Fig. 1, show a linear relationship between absorbance and glycerol concentration within the range of 3 to 17 γ. Amounts of glycerol as low as 0.2 γ gave measurable absorbance differences (approx. 0.01).

Known amounts of glycerol were added to several tubes after addi-

[2] The trichloracetic acid and enzyme were added to the control tube at the end of the incubation to avoid the slow hydrolysis of the monoolein that occurs at the low pH (1.8) which results upon the addition of the trichloracetic acid.

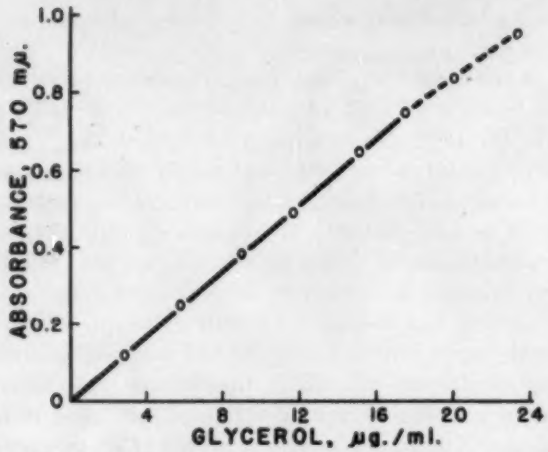

Fig. 1. Relation between glycerol concentration and absorbance at 570 mμ.

tion of the inactivating reagent and subsequently the glycerol was separated and measured. The results given below show recoveries from 96 to 100%.

Glycerol Present	*Glycerol Found*	*Recovery*
γ/ml	*γ/ml*	*%*
0.00	0.0	...
2.65	2.6	98
3.98	3.9	98
5.29	5.3	100
7.95	7.7	97
10.6	10.2	96

It was desirable to use the minimal amount of substrate necessary to give maximum activity, since the monoolein contributed to the blank readings in spite of the previous washing to which it had been subjected. This phenomenon has been observed by others when cleaving aqueous extracts of lipids with periodate (14, 16). The amount required was determined by varying the substrate concentration from 0.01% to 1.5% with a steapsin concentration of 12.5 γ per ml. The following results show a definite leveling effect above concentrations of 0.5% monoolein:

Substrate Concentration	*Glycerol Released*
%	*γ/ml*
0.01	4.1
0.05	8.5
0.1	10.2
0.5	13.7
0.8	14.6
1.0	14.4
1.5	14.6

On the basis of these data a substrate concentration of 1% was chosen for the remaining experiments. With this substrate concentration, cleavage of only 0.23% of the total substrate present was necessary to give an absorbance increase of 0.5.

The results from a typical trial in which the enzyme concentration was varied from 4.18 to 12.50 γ/ml at a 1% level of monoolein show that the glycerol produced was strictly proportional to the enzyme concentration (Fig. 2).

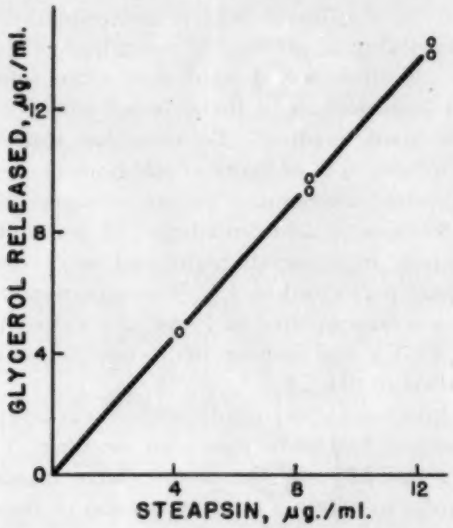

Fig. 2. Relation between steapsin concentration and glycerol released.

As a further test of the procedure, the activity exhibited by a single enzyme preparation toward three separately prepared emulsions was determined. The results, including the original absorbances (Table I), demonstrated good reproducibility between the emulsions as well as between duplicate trials for a given emulsion.

TABLE I

REPRODUCIBILITY OF THE LIPASE ASSAY

EMULSION NO.	ABSORBANCE				AVERAGE INCREASE	GLYCEROL RELEASED[a]
	Determinations		Blank			
	a	b	a	b		
						γ
1	0.755	0.750	0.208	0.208	0.544	14.0
2	0.750	0.750	0.202	0.197	0.550	14.2
3	0.758	0.780	0.205	0.203	0.565	14.6

[a] Released by 12.5 γ of steapsin.

Application of the Method to the Determination of Lipase Activity in Wheat Products

Wheat Products and Their Preparation. Samples of a hard red spring wheat mix and the principal flour and feed products were obtained from a commercial mill and defatted as described by Singer and Hofstee (20), by extracting five times with three volumes of low-boiling petroleum ether (Skelly-solv F).

Homogenates of the wheat products rather than water extracts of them were used for the lipase activity determinations in order to eliminate the possibility of incomplete extraction of the enzyme.

Preliminary experiments indicated that water-soluble materials were present in homogenates of the defatted wheat fractions which caused excessive blank readings. To overcome this difficulty, they were treated as follows: 1 g. of material was homogenized with 40 ml. of cold 95%-saturated ammonium sulfate solution (pH = 7.0), allowed to stand 30 minutes, and centrifuged for 5 minutes at approximately 10,000 r.p.m. in a Sorvall high-speed angle centrifuge.[3] The manipulations were performed at 3°C. The supernatant was decanted and the residue was homogenized in 10 ml. of a solution 0.004 *M* with respect to both EDTA and cysteine hydrochloride, which had previously been adjusted to pH 7.3.

Method. To give satisfactory results with wheat lipase, the method described for steapsin had to be somewhat modified. The pH of the reaction mixture was 7.35 and the samples were incubated at 30°C. for 1 hour. In order to obtain a clean separation of the flour particles and of the lipid and aqueous phases, it was necessary to add 1 ml. of low-boiling petroleum ether (Skelly-solv F) after addition of the inactivating reagent. The mixture was shaken vigorously, covered with aluminum foil, and centrifuged in an International No. 2 centrifuge (head No. 240) at approximately 3,000 r.p.m. for 20 minutes at 3°C. The lipid layer was then removed with a hypodermic syringe and 1 ml. of the aqueous phase was used for the determination of glycerol.

Effect of Enzyme Concentration, Temperature, pH, and Reaction Time. Before the activities of the different wheat fractions were compared, assays were made with a homogenate of red dog flour employing different enzyme concentrations, reaction times, pH values, and temperatures.

The relative amounts of hydrolysis at three enzyme concentrations and three time intervals with a 1% substrate concentration were:

[3] A later experiment, in which a sample of red dog was homogenized in 10 ml. of 0.004 M EDTA and 30 ml. of saturated ammonium sulfate, taken up in an appropriate amount of solution and assayed, resulted in a preparation with comparable activity. Homogenization was more readily accomplished with the slightly lower concentration of ammonium sulfate.

Enzyme Added[a] *ml.*	*Increase in Absorbance* 30 minutes	40 minutes	60 minutes
0.1	...	...	0.104
0.2	...	...	0.198
0.3	...	...	0.276
0.3	0.103	0.202	0.294

[a] The last row of values for 0.3 ml. enzyme solution was obtained on a different enzyme preparation from the others.

The influence of temperature and pH on the extent of hydrolysis is illustrated by the following data:

Temperature °C.	*Increase in Absorbance* pH 6.70	pH 7.35	pH 7.68
30	0.222	0.272	0.260
34	...	0.301	...
38	...	0.333	...

The changes in relative activity, as affected by pH and temperature, of red dog lipase acting on monoolein are very similar to those exhibited by purified wheat germ lipase acting on monobutyrin (20). Over the pH and temperature ranges investigated, the relative results of the studies are apparently superimposable.

The relative activities, expressed as absorbance, of four enzyme preparations from the same sample of defatted red dog flour were: 0.267, 0.276, 0.294, and 0.272. Thus, the activities of the individual preparations deviated by only about 5% of the mean value.

Relative Lipase Activities of Different Mill Fractions. Lipase activity determinations made on the various mill products from a hard red spring wheat mix are recorded in Table II, together with data obtained on sub-samples of the same fractions in the Quartermaster Food and Container Institute employing the procedure of Koch *et al.* (9).

Although the rank order of the lipase activities of the different fractions is similar for the two methods, the relative values are not in agreement. Thus, by the "glycerol method," the lipase activity of the shorts and germ fractions are 50 and 100 times that of the patent flour; by the procedure of Koch *et al.*, each of the fractions has only about four times the activity of the patent flour. The relative activities of the various mill fractions obtained by the glycerol assay method are in good general agreement with the limited data in the literature. Thus, Engel (7) found that wheat germ had the highest activity followed by the aleurone layer while the endosperm had negligible

TABLE II

LIPASE ACTIVITIES OF THE MILL PRODUCTS FROM A HARD RED SPRING WHEAT MIX OBTAINED BY THE GLYCEROL PROCEDURE AND BY THE METHOD OF KOCH *et al.* (9)

MILL FRACTION	MILL YIELD	CRUDE[a] PROTEIN	ASH[a]	LIPASE ACTIVITY[b]		PERCENT OF TOTAL ACTIVITY[e]	
				Glycerol Method[c]	Koch et al[d]	Glycerol Method	Koch et al
	%	%	%				
Patent flour	62.0	12.3	0.42	4	0.32	4.8	35.5
First clear	6.5	15.4	0.72	14	0.49	1.7	5.7
Second clear	3.5	16.5	1.60	77	0.78	5.2	4.9
Red dog	4.0	16.1	3.18	218	0.89	16.8	6.4
Shorts	12.0	15.2	4.59	236	1.12	54.3	24.0
Bran	11.5	15.0	5.99	60	1.09	13.3	22.4
Germ	0.5	29.0	4.47	403	1.21	3.9	1.1
Whole wheat	100.0	13.5	1.68	67	0.84		

[a] Expressed on 14.0% moisture basis.
[b] Expressed on original moisture basis (germ 8.2%, other fractions 10.5-12.1%).
[c] Lipase activity expressed as γ of glycerol released from glycerol monoleate by the lipases in 0.25 g. of the lipid-free fraction during 60 minutes at 30°C. and pH 7.35.
[d] Lipase activity is expressed as ml. of 0.1 N sodium hydroxide solution required to neutralize the fatty acids liberated from a butter fat emulsion by 4.0 g. of the wheat product during 60 minutes at 38°C. and pH 7.90.
[e] Percentage values are computed on the total activity of the mill fractions. The weighted values computed for whole wheat were 52 and 0.56 for the glycerol and Koch et al. procedures, respectively.

lipase activity. Miller and Kummerow (15), employing the method of Singer and Hofstee (19), which is based upon the manometric determination of carbon dioxide evolved from a bicarbonate buffer at pH 7.4 and 30°C. as a result of the liberation of free fatty acid from mono-n-butyrin, obtained lipase values of 1.4 and 132.4 for a sample of patent flour and wheat germ, respectively. These data indicate that the wheat germ had a lipase activity about 95 times greater than the patent flour. The increases in fat acidity observed by Cuendet *et al.* (2) upon storage of various mill streams at 14% moisture and 37.8°C. indicate a distribution of lipase in the various fractions which is in general agreement with that indicated by the glycerol procedure in the present series of samples.

The reason for the discrepancy between the relative activities of the different fractions by the titrimetric procedure of Koch *et al.* (9) as compared with the limited data available by other methods is not immediately apparent. The most likely possibilities are the differences in pH, in substrate, and in buffer concentration used in the different procedures. The researches of Thibodeau and Macy (27) and of Dirks *et al.* (5) show that the ionic strength of the buffers used may have a very marked influence on the values which are obtained with certain lipase preparations. In the procedure of Koch *et al.* (9) a very high buffer concentration is employed.

Discussion

The glycerol assay method is relatively simple, requires no special equipment, and is very sensitive. Quantities of glycerol from 0.2 to 18.0 γ/ml can be accurately measured directly and the upper limit can be extended by dilution.

The liberation of 0.2 γ of glycerol per ml. of a reaction mixture containing monoolein corresponds to an absorbance increment of approximately 0.01 and is equivalent to about 0.05 μl. of carbon dioxide produced or to 2.17×10^{-5} ml. of 0.1 *N* sodium hydroxide solution.

In its present form the procedure suffers the limitation of being directly applicable only to water-insoluble monoglyceride substrates; thus a two-phase system is required during the hydrolysis, since the lipase is water-soluble. The use of glycerides with long-chain fatty acids is advantageous as they more nearly approach the natural substrates than short-chain glycerides which have been widely used by various investigators. The method might be adapted to the use of water-soluble substrates through the use of suitable chromatographic or extraction technics to separate the glycerol and unchanged substrates.

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Literature Cited

1. Bradshaw, R. C. A. Lipolytic enzymes, with special reference to the baking industry. Food Sci. Abstracts **24:** 501–510 (1952).
2. Cuendet, L. S., Larson, E., Norris, C. G., and Geddes, W. F. The influence of moisture content and other factors on the stability of wheat flours at 37.8°C. Cereal Chem. **31:** 362–389 (1954).
3. Desnuelle, P. Hydrolyse enzymatique des triglycérides. Bull. soc. chim. biol. **33:** 909–923 (1951).
4. Desnuelle, P., and Naudet, M. Specific microcolorimetric method for the determination of formaldehyde; its application to the determination of glycerol and of ethylene- and propylene-glycol. Bull. soc. chim. **12:** 871–874 (1945). Chem. Abstracts **40:** 3699^5 (1946).
5. Dirks, B. M., Boyer, P. D., and Geddes, W. F. Some properties of fungal lipases and their significance in stored grain. Cereal Chem. **32:** 356–373 (1955).
6. Eegriwe, E. Reactions and reagents for the detection of organic compounds. Z. anal. Chem. **110:** 22–25 (1937). Chem. Abstracts **31:** 8442^2 (1937).
7. Engel, C. The distribution of enzymes in resting cereals. III. The distribution of esterase in wheat, rye, and barley. Biochim. Biophys. Acta **1:** 278–279 (1947).
8. Fodor, P. Inactivation studies on pancreatic lipase. III. The inactivation effect produced by substrate homologs. Arch. Biochem. Biophys. **35:** 311–320 (1952).
9. Koch, R. B., Felsher, Augusta R., Burton, T. H., and Larsen, R. A. A rapid method for the determination of cereal lipase activity. Cereal Chem. **31:** 113–120 (1954).

10. KRITOVICH, V. I., SOKOLOVA, A. I., and USHAKOVA, E. N. The stable moisture content of grain and its effects on the lipase action. Compt. rend. acad. sci. U.R.S.S. **27:** 701–704 (1940). Chem. Abstracts **35:** 8126 (1941).
11. LAMBERT, MARGUERITE, and NEISH, A. C. Rapid method for estimation of glycerol in fermentation solutions. Can. J. Research **B28:** 83–89 (1950).
12. LONGENECKER, H. E. Esterases. *In* Enzymes and their role in wheat technology, ed. by J. A. Anderson. Chap. IV. Interscience: New York (1946).
13. MACFAYDEN, D. A. Estimation of formaldehyde in biological mixtures. J. Biol. Chem. **158:** 107–133 (1945).
14. MATTSON, F. H., BENEDICT, J. H., MARTIN, J. B., and BECK, L. W. Intermediates formed during the digestion of triglycerides. J. Nutrition **48:** 335–344 (1952).
15. MILLER, B. S., and KUMMEROW, F. A. The disposition of lipase and lipoxidase in baking and the effect of their reaction products on consumer acceptability. Cereal Chem. **25:** 391–398 (1948).
16. POHLE, W. D., and MEHLENBACHER, V. C. A modification of the periodic acid method for the determination of monoglycerides and free glycerol in fats and oils. J. Am. Oil Chemists Soc. **27:** 54–56 (1950).
17. RONA, P., and LASNITZKI, A. Eine Methode zur Bestimmung der Lipase in Körperflüssigkeiten und im Gewebe. Biochem Z. **152:** 504–522 (1924).
18. SHIMIZU, M. Pancreatic lipase. I. The active centers. J. Japan Biochem. Soc. **24:** 205–209 (1952–1953). (Original not seen.) Chem. Abstracts **47:** 12453[i] (1953).
19. SINGER, T. P., and HOFSTEE, B. H. J. Studies on wheat germ lipase. I. Methods of estimation, purification, and general properties of the enzyme. Arch. Biochem. **18:** 229–243 (1948).
20. SINGER, T. P., and HOFSTEE, B. H. J. Studies on wheat germ lipase. II. Kinetics. Arch. Biochem. **18:** 245–259 (1948).
21. SIZER, I. W. Effects of temperature on enzyme kinetics. *In* Advances in enzymology, Vol. 3, ed. by F. F. Nord and C. H. Werkman, pp. 35–62. Interscience: New York (1943).
22. STEDMAN, E., and STEDMAN, ELLEN. The relative choline-esterase activities of serum and corpuscles from the blood of certain species. Biochem. J. **29:** 2107–2111 (1935).
23. SULLIVAN, BETTY. Esterases in relation to milling and baking. *In* Enzymes and their role in wheat technology, ed. by J. A. Anderson. Chap. V. Interscience: New York (1946).
24. SULLIVAN, BETTY, and HOWE, MARJORIE. Lipases of wheat. I. J. Am. Chem. Soc. **55:** 320–324 (1933).
25. SUMNER, J. B., and MYRBÄCK, K. The enzymes. Vol. I, Part 1. Academic Press: New York (1950).
26. SUMNER, J. B., and SOMERS, G. F. Chemistry and methods of enzymes. Academic Press: New York (1953).
27. THIBODEAU, R., and MACY, H. Growth and enzyme activity of *Penicillium roqueforti*. Minn. Agr. Expt. Sta. Tech. Bull. 152 (1942).
28. WIDHE, T., and ONSELIUS, T. Lipolysis in doughs made from various cereal flours. Cereal Chem. **26:** 393–398 (1949).

EXTRACTION OF LIPIDS FROM WHEAT PRODUCTS[1]

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ABSTRACT

Of a number of solvents tried, water-saturated n-butyl alcohol was most effective in extracting lipid material from ground whole wheat, flour, bran, and gluten. This supports the observation of Morton (Nature **166**: 1092–1095 (1950)) that this solvent is outstandingly efficient in removing lipid from biological materials. For example, extraction of three wheat flours with water-saturated n-butyl alcohol at room temperature removed an average of 1.62% chloroform-soluble lipids compared to 1.43% extractable by the next-best solvent, hot absolute ethyl alcohol in a Soxhlet extractor for 18 hours. With bran and whole wheat, differences were larger. From three bran samples, n-butyl alcohol removed an average of 6.19% lipid; ethyl alcohol, 5.66%. Small amounts of nonlipid materials also were present in the water-saturated butyl alcohol extracts; more were found in extracts of flour and bran than in extracts from gluten.

Following the n-butyl alcohol extractions, glutens could be washed from the extracted flours and extracted glutens could be wetted to form a rubbery mass. Therefore, in contrast to other effective lipid extractants, the water-saturated butyl alcohol treatment does not entirely destroy the cohesive properties of the wheat proteins.

The extraction of lipid material from cereal products presents difficulties from both an analytical and a preparative viewpoint. In order to remove all lipid from flour, macaroni, bread, and other cereal products, the methods of the Association of Official Agricultural Chemists and the American Association of Cereal Chemists prescribe treatment of the sample with hydrochloric acid and extraction with ethyl alcohol-ether mixtures. Other procedures employing treatments of varying degrees of severity have been used for specific purposes by several workers, including treatment of wheat gluten with ethyl alcohol and ammonia (20) and addition of cationic detergent to baked foods (12). Such procedures obviously denature the protein constituents or degrade the proteins and the lipids of cereal products and are unusable for preparative work. Extraction with fat solvents such as diethyl ether and petroleum ether, which do not appear to injure other constituents, does not remove all lipid (7, 20). Procedures which would extract all lipids from flour and gluten without damaging other components therefore have been needed to permit preparation of flour constituents in a lipid-free state and investigation of the role of flour lipids in baking performance.

The approach to these problems employed in the work described

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here was suggested by the reports of Morton (16) and Hurst (11) that water-saturated normal butyl alcohol is peculiarly effective in disrupting protein-lipid complexes in biological materials from various sources without damage to protein properties. Morton was in fact primarily concerned with the release of enzymes in a water-soluble, active form from lipoprotein particles. Also water-saturated n-butyl alcohol was recommended for the extraction of wheat and flour pigments in 1938 by Binnington, Sibbitt, and Geddes (4) and has been used extensively for that purpose. This use, together with the observations of Morton, suggested that extraction with water-saturated n-butyl alcohol might provide a rapid method for complete removal of lipids from cereal products, without damage to protein properties.

The present paper is concerned primarily with the completeness with which water-saturated n-butyl alcohol removes lipid constituents from several wheat products—that is, with the analytical rather than the preparative possibilities of the procedure. Some information, however, on the composition of lipids and a few observations on the properties of the lipid-free flours and glutens are included.

Materials and Methods

Source and class or variety of the wheat from which samples were prepared are indicated in the tables; all were from sound, clean grain. Flour and bran samples were from experimental millings. Whole wheat and bran samples were ground in a Wiley mill to pass a 40-mesh screen before extraction of lipid.

Gluten samples were washed out with distilled water, except the sample from Elgin flour. For this sample 0.1% phosphate buffer (pH 6.8) was used until the gluten had collected in an elastic mass; washing then was completed with distilled water. Unless otherwise indicated, gluten samples were dried by lyophilizing in order to obtain a porous structure.

When Soxhlet extraction equipment was used, the samples of 2 or 3 g. were wrapped in thin layers in filter paper, and extraction was carried out for 16–20 hours. In the case of water-saturated n-butyl alcohol extraction at room temperature, the sample (usually 2 g. of gluten or 5 g. of other products) was placed in a centrifuge tube and 10 ml. of the solvent per g. of sample were added. Solvent and sample were thoroughly mixed by gentle shaking, and the tube then was allowed to stand with occasional agitation. At the end of the extraction period, the tube was centrifuged, and from the clear supernatant extract a portion (10 or 25 ml.) was withdrawn. Overnight extraction was routinely employed for analytical purposes. Shorter times are adequate, as

shown in a following section, and were used for preparative work as indicated in the text.

The entire Soxhlet extract or the portion of n-butyl alcohol extract was concentrated under a gentle air stream on a steam bath almost to dryness. Chloroform then was added and the chloroform solution filtered through medium-porosity sintered glass, covered with analytical grade Filter-Aid, into a tared beaker. After evaporation of chloroform, the lipid residues were dried in a vacuum oven at 50°C. and weighed.

Total phosphorus was determined by the method of Allen (1); total nitrogen by macro- or micro-Kjeldahl procedures (3, 24). Amide nitrogen was determined as described previously (14).

Results

Comparison of Butyl and Ethyl Alcohols. To obtain reasonably complete removal of lipids from flours and lyophilized glutens, extraction with absolute ethyl alcohol for 16–20 hours in a Soxhlet apparatus had been adopted as a standard procedure in earlier work in this laboratory. In comparisons with other solvents and extraction methods, the ethyl alcohol-Soxhlet procedure had been found to remove at least as much lipid as any other combination tried. This experience is consistent with that of others, with respect to biological materials in general (13) and cereal products as a class (9, 21, 23).

Preliminary experiments indicated that extraction with n-butyl alcohol saturated with water removed as much lipid (chloroform-soluble material) from wheat flour at room temperature as the ethyl alcohol-Soxhlet procedure. Lipid removal was quite rapid. The slowest extraction observed was from a bran sample, although in the case of a gluten dried in a vacuum oven at 45°C. and ground to pass a 40-mesh screen, extraction was not rapid in the first few minutes of contact with the water-saturated n-butyl alcohol. The amounts of lipid obtained at various time intervals were as follows:

Extraction Time	*Lipid from Bran*	*Lipid from Gluten*
	%	%
5 minutes	4.34	2.01
30 minutes	4.60	5.60
1 hour	4.99	6.91
2 hours	5.29	7.39
4 hours	5.60	7.38
17 hours	5.58	7.52

Complete extraction of other products was obtained more rapidly; 30 minutes were adequate with flour samples.

More extensive comparison of the two procedures then was made

TABLE I

AMOUNTS OF LIPID EXTRACTED FROM WHEAT PRODUCTS BY WATER-SATURATED N-BUTYL ALCOHOL AND BY ETHYL ALCOHOL

PRODUCT	WHEAT VARIETY	YEAR	ORIGIN	ETHYL ALCOHOL LIPID	N-BUTYL ALCOHOL	
					Lipid [a]	Non-Lipid [a]
				%	%	%
Whole wheat	Durum	1952	N. D. composite	3.05	3.70	0.54
	Baart	1951	Pac. N.W. composite	2.70	3.30	0.51
	Elgin	1950	Pullman, Washington	2.48	2.94	0.52
Flour	Durum	1953	N. D. composite	1.41	1.74	0.26
	Baart	1951	Pac. N.W. composite	1.50	1.61	0.31
	Elgin	1948	Pendleton, Ore.	1.37	1.51	0.22
Gluten	Durum	1953	N. D. composite	7.36	7.45	0.12
	Baart	1951	Pac. N.W. composite	7.06	7.16	0.14
	Elgin	1948	Pendleton, Ore.	12.10	12.24	0.19
Bran	Baart	1952	Pac. N.W. composite	5.44	6.14	1.47
	Elmar	1952	Pullman, Washington	5.53	6.03	1.73
	Rex	1952	Pullman, Washington	6.01	6.40	1.49

[a] Extracted material soluble in chloroform.

with some additional wheat products; the results are given in Table I. The water-saturated n-butyl alcohol consistently removed more lipid than the ethyl alcohol. The differences with the gluten samples are small, but rather large with whole wheat and bran. The amounts of material extracted by the wet butyl alcohol but not soluble in chloroform are also shown in Table I. With the exception of the gluten extracts, a rather high proportion of chloroform-insoluble material was obtained, amounting to 16–25% of the lipid weights.

In those cases in which butyl alcohol extracted appreciably more lipid than ethyl alcohol, differences in composition of the lipids might be expected. Phosphorus and nitrogen contents (Table II) differ significantly in some instances; for example, the nitrogen contents of the butyl alcohol extracts of the whole wheats are higher than those of the ethyl alcohol extracts. In general, however, the differences are neither sufficiently consistent nor large enough to be informative.

Residual Fatty Acid Content. To provide further evidence regarding the completeness of extraction of lipids from wheat products, the method used by Folch *et al.* (8) with brain tissue was used. The residual materials, after n-butyl or ethyl alcohol extraction, were hydrolyzed with 20% sodium hydroxide, under reflux for 16–20 hours, and brought to pH 2 by addition of hydrochloric acid; the acidified digests were extracted with petroleum ether. The petroleum ether-soluble materials (assumed to be fatty acids) set free by the hydrolysis were weighed (Table III). Very little fatty acid was released following either

TABLE II

PHOSPHORUS AND NITROGEN CONTENT OF LIPIDS FROM WHEAT PRODUCTS

PRODUCT	WHEAT VARIETY	YEAR	ORIGIN	PHOSPHORUS IN LIPID EXTRACTED WITH:		NITROGEN IN LIPID EXTRACTED WITH:	
				Ethyl Alcohol	Butyl Alcohol	Ethyl Alcohol	Butyl Alcohol
				%	%	%	%
Whole wheat	Durum	1952	N. D. composite	0.47	0.46	0.33	0.53
	Baart	1951	Pac. N.W. composite	0.60	0.57	0.45	0.77
	Elgin	1950	Pullman, Wash.	0.53	0.54	0.38	0.68
Flour	Durum	1953	N. D. composite	0.53	0.74	0.59	0.72
	Baart	1951	Pac. N.W. composite	0.58	0.68	0.57	0.62
	Elgin	1948	Pendleton, Ore.	0.45	0.50	0.49	0.49
Gluten	Durum	1953	N. D. composite	0.53	0.51	0.49	0.37
	Baart	1951	Pac. N.W. composite	0.71	0.61	0.51	0.41
	Elgin	1948	Pendleton, Ore.	0.73	0.66	0.56	0.35
Bran	Baart	1952	Pac. N.W. composite	0.55	0.49	0.42	0.48
	Elmar	1952	Pullman, Wash.	0.57	0.51	0.44	0.44
	Rex	1952	Pullman, Wash.	0.53	0.51	0.38	0.43

the butyl or ethyl alcohol treatments, indicating that essentially complete extraction of lipid had been obtained.

Other Extraction Procedures. The difficulty of obtaining complete lipid extraction from cereal products has been recognized in the development of the official methods of the Association of Official Agricultural Chemists in the United States, and by the Cake and Flour Confectionery Order in Great Britain, which require treatment of samples with hydrochloric acid prior to extraction with an ethyl alcohol-ether mixture. These methods are quite similar; n-butyl alcohol extraction was compared only with the latter, as originally described (17) and as modified by Morgan and Rawlings (15) to use a digestion temperature of 50°C. rather than 80°C. Results obtained with a lyophilized gluten sample were: n-butyl alcohol extraction,

TABLE III

AMOUNTS OF PETROLEUM ETHER-SOLUBLE MATERIALS SET FREE BY ALKALINE HYDROLYSIS OF WHEAT PRODUCTS FOLLOWING N-BUTYL ALCOHOL OR ETHYL ALCOHOL EXTRACTION

Sample	Butyl Alcohol-Extracted Residue	Ethyl Alcohol-Extracted Residue
	%	%
Mida flour	0.00	0.07
Pawnee flour	0.01	0.02
Durum wheat	0.03	0.08
Mida gluten	0.04	0.10

11.1% lipid; 80°C. acid digestion, 9.6%; 50°C. acid digestion, 8.8%. It is apparent that with gluten in the physical state obtained by lyophilizing, the n-butyl alcohol extraction was more effective than the acid-alcohol treatments employed.

Sugar Contents of the Lipid Extracts. The presence of sugars in wheat lipid extracts has been reported by Sullivan *et al.* (22). Because the butyl alcohol extraction procedure consistently had yielded more chloroform-soluble material than the ethyl alcohol extraction, it was thought that the solvent properties of the water-saturated butyl alcohol might be such that the additional material would be made up largely of sugars associated with sufficient lipid to account for their solubility in chloroform. Preliminary analyses showed 3% or more sugar (as glucose) in the extracts of two flours. Some additional information then was collected as shown in Table IV. In an effort to dis-

TABLE IV

FLOUR, WHEAT, AND GLUTEN LIPID FRACTIONS AND THEIR SUGAR CONTENTS
(Values are given on the basis of original sample weight)

SAMPLE	ORIGINAL EXTRACTANT	EXTRACT IN:		SUGARS IN:	
		Petroleum Ether	50% Aqueous Ethyl Alcohol	Petroleum Ether Solubles	50% Aqueous Ethyl Alcohol Solubles
		%	%	%	%
Mida flour	n-Butyl alcohol	1.03	1.11	0.046	0.20
	Ethyl alcohol	1.21	0.21	0.037	0.035
Pawnee flour	n-Butyl alcohol	1.06	1.10	0.047	0.21
	Ethyl alcohol	1.34	0.25	0.027	0.035
Durum wheat	n-Butyl alcohol	2.53	1.73	0.092	0.62
	Ethyl alcohol	2.71	0.26	0.023	0.033
Mida gluten	n-Butyl alcohol	8.88	1.63	0.39	0.10
	Ethyl alcohol	9.85	0.65	0.108	0.067

tinguish between free sugars and those combined with lipid, a procedure similar to that used by Scholfield and Dutton (18) with soybean phospholipids was employed; the n-butyl or ethyl alcohol extract was taken up in petroleum ether and extracted with 50% aqueous ethyl alcohol. Soluble material from each phase was recovered and reducing sugars were determined after an arbitrarily chosen hydrolysis treatment (7 to 8 hours' refluxing in 2 *N* sulfuric acid).

The partition between petroleum ether and 50% aqueous ethyl alcohol differs markedly for the butyl and ethyl alcohol extracts, with the butyl alcohol extracts showing much more tendency to go into the aqueous ethyl alcohol phase. This was particularly evident in the

cases of the butyl alcohol extracts of the flours, more than half of the total extract being recovered from the aqueous ethyl alcohol. The aqueous ethyl alcohol-soluble solids had the appearance of fatty material, and apparently contained only 15–20% of sugars. These figures for sugar content are given primarily for comparative purposes, however, because the hydrolysis procedure may not have given maximum yields. The butyl alcohol-extract fractions did yield more sugar than the ethyl alcohol-extract fractions, but the additional sugar accounts for one-half at most of the differences in total amount of lipid extracted. It appears likely that compounds or complexes containing sugars, rather than sugars alone, were extracted by the n-butyl alcohol. This is suggested also by the earlier observations of Antener and Högl (2), who reported that the lipids of wheat germ occur in complex with both carbohydrate and protein; they reported separation of a lipid fraction containing 49% phospholipid and 43% sucrose.

Properties of Extracted Flours and Glutens. It was of particular interest in view of Morton's observations to determine whether the gluten proteins, after n-butyl alcohol extraction, retained their elastic and cohesive properties which are considered to be the basis of their usefulness in baking.

Glutens could be recovered after butyl alcohol extraction by washing with dilute buffer or sodium chloride solutions, which removed most of the alcohol and left a coherent gluten mass. The glutens recovered were fairly elastic but less extensible than unextracted glutens.

The solubility of gluten after extraction with n-butyl alcohol was compared with that of ordinary gluten in several solvents commonly used for gluten dispersion. For this purpose butyl alcohol was removed from the gluten by washing with wet ethyl ether to make certain that no loss of protein occurred; the ether then was evaporated in an air stream at room temperature and finally in a vacuum oven at 50°C. Results are given in Table V. A somewhat smaller proportion of gluten protein can be dispersed after extraction, perhaps indicative of some denaturation.

Protein recovered from the dispersions was compared with respect to amide nitrogen content; these results also are presented in Table V. No consistent difference in amide nitrogen content of the protein dispersed from the unextracted and butyl alcohol-extracted glutens was found.

After butyl alcohol was washed from extracted *flours* with wet ether and the ether was removed by evaporation in an air stream and finally in a vacuum oven at 50°C., gluten could be washed from the flours in the usual way in most instances. However, the odor of butyl

TABLE V

EFFECT OF N-BUTANOL EXTRACTION ON SOLUBILITY OF GLUTEN AND ON THE AMIDE CONTENT OF THE SOLUBLE FRACTION

Gluten	Solvent	Gluten Nitrogen Soluble	Amide Nitrogen of Total Nitrogen in Soluble Gluten Protein
		%	%
U[a]	60% ethanol	58	25.1
E[a]		50	24.5
U	0.1 *M* acetic acid	88	23.0
E		71	23.8
U	8% sodium salicylate	84	24.2
E		69	24.2

[a] U, gluten not treated; E, gluten extracted with n-butyl alcohol.

alcohol could be detected as soon as the flour was wetted and mixing into a dough was begun. Some butyl alcohol therefore was bound so firmly to flour constituents that it could not be removed at 50°C. in vacuum; but it was displaced by water. Similarly, it was found that when the vacuum-dried flour was exposed to an atmosphere of high relative humidity until the flour moisture content reached 20% or more and the flour was again dried in the vacuum oven, all butyl alcohol apparently was removed, as judged by lack of odor upon mixing a dough.

A few observations have been made on the bread-baking behavior of the extracted flours (freed of residual butyl alcohol as indicated in the preceding paragraph). In the first baking trials, micro doughs were mixed in a recording mixer; the butyl alcohol-extracted flour doughs, lipid-free or with flour lipid added back, required a fourfold increase in mixing time to reach maximum development as compared to the untreated flour. A presoak procedure then was used; all ingredients except yeast were combined and held for 1 hour. Yeast was then added and the doughs mixed to maximum resistance. The extracted flour doughs still required twice as long to develop as the control. However, with a Mida wheat flour, a 3-hour straight dough procedure and 3% shortening in the formula, micro loaf volumes were: control flour, 195 ml.; butyl alcohol-extracted, 138 ml.; and butyl alcohol-extracted plus extract in the amount originally present in the flour, 181 ml. Butyl alcohol extraction thus resulted in a pronounced loss in loaf volume, but about two-thirds of the loss was regained by replacement of the extracted material. With two other flours, regains of loaf volume were smaller. The significance to be attributed to these loaf volume differences must be questioned in view of the abnormal mixing

behavior observed with extracted flour samples even with lipid added back. Insofar as the proteins are concerned, however, recovery of loaf volume upon addition of lipid to the extent observed with the Mida flour suggests that with careful handling, the proteins can retain to a large degree those properties essential for bread baking, and that little denaturation need occur.

Discussion

The ease with which lipid can be removed from the wheat products examined suggests that water-saturated n-butyl alcohol might prove to be a useful extractant for the analytical determination of total lipids in cereal products in general. The presence of some material perhaps not strictly lipid in nature in the butyl alcohol extracts might require further treatment, either distribution between suitable solvents or degradation to some degree with acid or alkali, to separate lipid material. However, this would appear to be as simple as the procedures now employed in an attempt to extract all lipid from cereal products with other solvents.

The possibilities for investigation of the role of lipids in the baking properties of flours are somewhat encouraging. This problem has been of interest to many investigators. For example, Working suggested in 1924 (25) that phospholipids affect gluten quality. Blish (5) reported the separation of fractions of the gluten proteins containing up to 44% lipid; disruption of the lipid-protein combination by ethyl alcohol was considered responsible for profound changes in gluten properties. Sinclair and McCalla (19) concluded that the more insoluble lipids were "essential for the formation and maintenance of the characteristic dough and gluten structure found with wheat flour." More recently, Coppock *et al.* (6) reported the separation of a phospholipid fraction from flour oil which exerted a definite effect on baking properties at a level of 0.003% flour weight (presumably in the absence of other fats). Hess (10) has made extensive physical observations on fractions separated from flours, by methods avoiding the use of water, and also has come to the conclusion that the flour lipids play an important role in determining the nature of doughs and the baking properties of flours.

It seems clear that the flour lipids modify the behavior of doughs and glutens so markedly that certain lipids must be necessary to approach normal baking properties. If flours or glutens can be processed so that the proteins are undamaged by the n-butyl alcohol treatment, the effects of flour lipids on protein properties and baking behavior could be determined by comparison with lipid-free materials, rather

than with materials only partially freed of lipid, in which those lipid materials most intimately combined with protein are still present.

Acknowledgments

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Literature Cited

1. Allen, R. J. L. The estimation of phosphorus. Biochem. J. **34:** 858–865 (1940).
2. Antener, I., and Högl, O. Studien uber die Phosphatide in Weizenkamen. Mitt. Gebiete Lebensm. Hyg. **38:** 226–244 (1947).
3. Association of Official Agricultural Chemists. Official methods of analysis (7th ed.). The Association: Washington, D. C. (1950).
4. Binnington, D. S., Sibbitt, L. D., and Geddes, W. F. A comparative study of the utility of various commercially available organic solvents for the determination of wheat and flour pigments. Cereal Chem. **15:** 119–132 (1938).
5. Blish, M. J. The wheat flour proteins. Cereal Chem. (Suppl.) **13** (No. 5): 16–23 (1936).
6. Coppock, J. B. M., Cookson, M. A., Laney, D. H., and (in part) Axford, D. W. E. The role of glycerides in baking. II. The influence of flour oils on the behavior of glycerinated fats in baking, and the effect of natural monoglycerides present in flour oils and baking fats on the pharmacological desirability of using glycerinated fats in baked products. J. Sci. Food Agr. **5:** 19–26 (1954).
7. Dill, D. B. The composition of crude gluten. Cereal Chem. **2:** 1–11 (1925).
8. Folch, J., Ascoli, I., Lees, M., Meath, J. A., and LeBaron, F. N. Separation of lipide extracts from brain tissue. J. Biol. Chem. **191:** 833–841 (1951).
9. Herd, C. W., and Amos, A. J. Fat: Its estimation in wheaten products. Cereal Chem. **7:** 251–269 (1930).
10. Hess, K. Protein, Kleber und Lipoid in Weizenkorn und Mehl. Kolloid Z. **136:** 84–99 (1954).
11. Hurst, H. Asymmetrical behavior of insect cuticle in relation to water permeability. Discussions Faraday Soc. **3:** 193–210 (1948).
12. Kiermeier, F., and Patschky, A. Die bestimmung des gebundenen Fettes in Lebensmitteln mit Hilfe von Zephirol. Z. Lebensm.-Untersuch.-u.-Forsch. **90:** 98–100 (1950); *from* Food Science Abstracts **24:** 51 (1952).
13. Meara, M. L., *in* Modern Methods of Plant Analysis, ed. by K. Paech and M. V. Tracey. Vol. II, pp. 365–366. Springer, Berlin (1955).
14. Mecham, D. K., and Olcott, H. S. Effect of dry heat on proteins. Ind. Eng. Chem. **39:** 1023–1027 (1947).
15. Morgan, R. H., and Rawlings, H. W. The determination of fat in sweetened foods. Analyst **76:** 161–166 (1951).
16. Morton, R. K. Separation and purification of enzymes associated with insoluble particles. Nature **166:** 1092–1095 (1950).
17. Nicholls, J. R., Colgate, R. T., Fraser, L. S., and Hughes, E. B. Methods of analysis for the purposes of the cake and flour confectionery (control and maximum prices) order, 1942 (S. R. & O. No. 2103 of 1942). Analyst **68:** 48–50 (1943).
18. Scholfield, C. R., and Dutton, H. J. Separations of soy bean inositide fractions of low partition coefficient. J. Biol. Chem. **208:** 461–469 (1954).
19. Sinclair, A. T., and McCalla, A. G. The influence of lipoids on the quality and keeping properties of flour. Can. J. Research **C15:** 187–203 (1937).
20. Sullivan, Betty, and Near, Cleo. Chemical constituents which influence gluten quality. Ind. Eng. Chem. **19:** 159–161 (1927).
21. Sullivan, Betty, and Near, Cleo. Changes of crude lipides of wheat on storage. Ind. Eng. Chem. **25:** 100–103 (1933).

22. SULLIVAN, BETTY, HOWE, MARJORIE, SCHMALZ, F. D., and ASTLEFORD, G. R. The action of oxidizing and reducing agents on flour. Cereal Chem. 17: 507–528 (1940).
23. TUCKER, I. W. Effect of flour lipids on recovery of gluten from hard and soft wheat flours by the use of sulfite solutions. Cereal Chem. 23: 217–224 (1946).
24. WHITE, L. M., and SECOR, GERALDINE E. Micro-Kjeldahl determination of nitrogen in gramicidin and tryptophan. Comparison of Gunning-Arnold-Dyer and Friedrich methods. Ind. Eng. Chem., Anal. Ed. 18: 457–458 (1946).
25. WORKING, E. B. Lipoids, a factor influencing gluten quality. Cereal Chem. 1: 153–158 (1924).

EFFECT OF WHEAT VARIETY AND GROWTH LOCATION UPON THE SPECIFIC VOLUME OF FLOUR[1]

R. H. HARRIS[2] AND G. H. BRUNER[2]

ABSTRACT

An apparatus for determining specific volume of flour is described. Very significant differences were found between the specific volumes of flour from ten wheat varieties grown at six stations in North Dakota in 1954. Very significant, although relatively smaller, differences were also found between stations at which the wheats were grown. The range between varieties was 0.45 or 30% in specific volume and the range between stations was 0.18 or 17% in specific volume. Wheats classed as unsatisfactory in milling characteristics had the largest flour volume.

Abnormally soft and fluffy types of hard red spring wheat flours had been observed as early as 1945 when a variety developed in South Dakota and known as Spinkota was first tested for milling and baking quality in this department. After five years of testing, this variety was discarded. In recent years, with hard red spring wheat hybrids being produced to combat damage from 15B stem rust, it again became apparent in some instances that the resultant flour tended to be "soft" and "fluffy" and more bulky than normal hard red spring wheat flour. Harris and Scott (1) showed that two new hybrids were significantly different in particle size distribution from wheats which have been approved for farm growth in the hard red spring wheat area.

The production of a light fluffy flour on a mill which is flowed and spouted for a free-flowing, granular type of flour tends to cause trouble and chokes in the flour spouts, resulting in nonuniform flour and delay in production.

This study was initiated to determine the specific volume of hard red spring wheat flour, and ascertain the effects of variety and location

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of growth upon this property.

Materials and Methods

Four flours used for a preliminary investigation to ascertain whether differences in specific volume existed between varieties and to develop a method were semicommercially milled from wheats obtained by the Northwest Crop Improvement Association in 1954. These included a range from poor and unsatisfactory, as PW 36, to very satisfactory, as Mida and Lee. The flours used for the chief part of the study were long patents experimentally milled on an Allis-Chalmers mill, using a fixed procedure, from wheats comparably grown in North Dakota in 1954. The samples were free from damage except from stem rust and drought. Three flours milled in the micro mill from nursery samples were also included to furnish information on flour from new hybrid wheats grown in nursery rows.

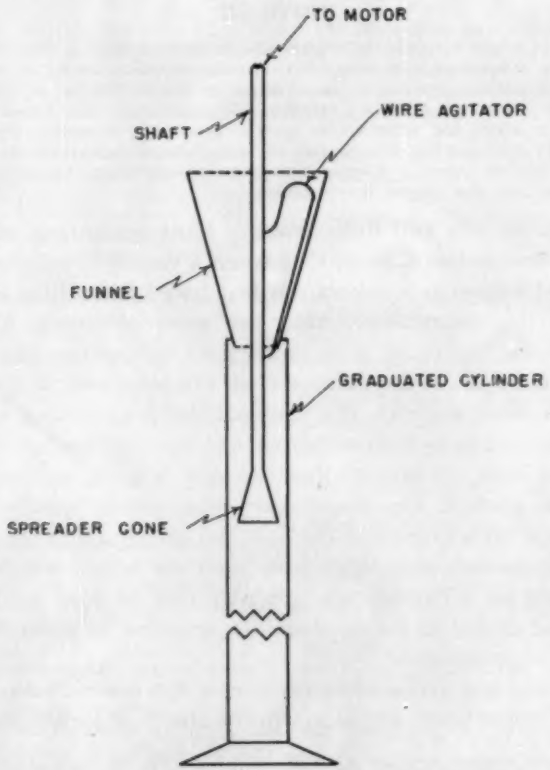

Fig. 1. Cut-away view of flour volume measuring apparatus. The spreader cone is drawn up to close the funnel while the sample is poured into the funnel.

Figure 1 shows the apparatus used for ascertaining flour volume. A bright tin funnel having no spout and of approximately 300-ml. capacity was used. The sides of the funnel were inclined at an angle of 75° from the horizontal. A spreader was required to insure even distribution and flow of the flours from the funnel into the measure. This spreader was a cone with an angle of 75° and approximately 1 in. in diameter attached to a rod ¼ in. in diameter. The apex was positioned just below the funnel orifice. The spreader was maintained at a constant suitable height in the 250-ml. graduated cylinder used to measure the flour volume. The agitator, necessary to prevent clogging and choking of the funnel opening, was a wire wing fastened to the shaft and shaped so that it just cleared the sides of the funnel. A geared-head motor was used to turn the agitator and spreader at a constant speed of 10 r.p.m. This speed was found to be the most satisfactory and must be held constant to obtain consistent results.

Seventy-five grams of flour were used for a determination, as this weight could be accommodated in a 250-ml. graduated cylinder. A larger cylinder or a smaller weight of flour would reduce the accuracy of the test. The flour was sifted four times through an ordinary hand flour sifter immediately before testing. There was no further increase in volume after this number of siftings. The flour was also sifted after each determination, using the same sifter. Ten determinations were made on each sample and the average of these was taken as the flour volume of the sample.

Results

The term specific volume as used in this paper means the volume occupied by 1 g. of flour when allowed to fall freely into the cylinder.

The results from the preliminary investigation with semicommercially milled flours are shown in Table I, with the identity of the original wheats from which the flours were milled. Flour from hybrid PW 36 had the largest volume, while flour from Willet was next in volume followed by flour from Mida. Lee had the smallest flour volume. The standard error of a single determination was 1.64, and the standard error of the mean of ten determinations was 0.55.

The differences between varieties were extremely significant, showing that the method was capable of differentiating wheat varieties with respect to their flour volume, and indicated that wheats known to be more difficult to mill than standard hard red spring varieties, such as PW 36, and which yielded soft flours, would probably give increased flour volume.

Table II is an analysis of variance of the data obtained from ten

wheats grown at six stations. Differences between varieties and stations are very significant. The effect of varieties (Fig. 2) is much larger than that due to stations (Fig. 3), chiefly because of the two varieties, Willet and Minn. 2854, which had extremely large flour volumes. Mida also had a larger flour volume than the seven remaining wheats, but was significantly below that of Willet and Minn. 2854. All three would be classed as softer wheats for milling than the others, and Harris and Scott (1) showed that Willet and Minn. 2854 were significantly different from the others in flour particle size distribution. Dickinson and Edgely produced wheats yielding smaller flour volumes than the other four stations represented. These differences should not be sufficiently great to cause concern.

It is apparent that flour from Willet and Minn. 2854 would require more volume in milling equipment since the flour is aerated in

TABLE I

REPLICATE FLOUR VOLUMES OBTAINED FOR FOUR COMMERCIALLY MILLED FLOURS

	WHEAT VARIETY			
	PW 36	Willet	Mida	Lee
	ml.	*ml.*	*ml.*	*ml.*
	215	190	184	164
	212	196	185	167
	213	195	185	166
	211	194	186	165
	213	193	183	166
	213	192	186	168
	213	195	186	166
	215	193	188	168
	216	190	186	166
	216	194	185	168
Mean	213.7	193.2	185.4	166.4
Mean specific flour volume, ml/g	2.85	2.57	2.47	2.22

TABLE II

ANALYSIS OF VARIANCE OF FLOUR VOLUME DATA

Source of Variation	Degrees of Freedom	Variance	F (Replicates for Error)	F (Varieties × Stations for Error)
Between varieties	9	1.7615	2202**	100.6**
Between stations	5	0.5847	731**	33.4**
Interaction				
Varieties × Stations	45	0.0175	22**	
Replications	540	0.0008		
Total	599			

** indicates that the 1% level of significance was exceeded.

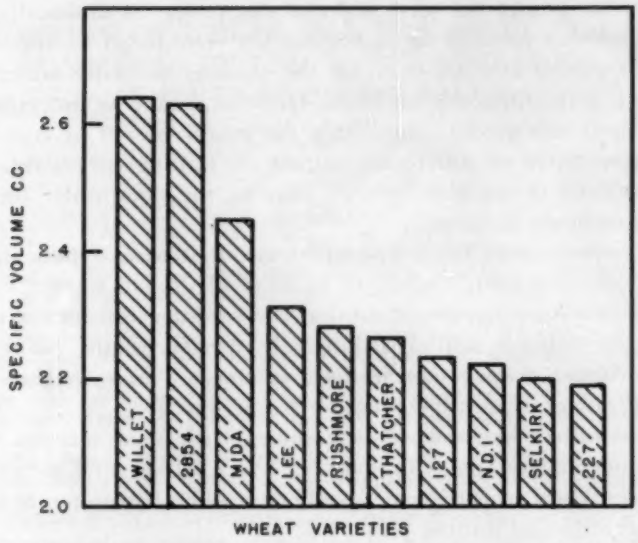

Fig. 2. Specific flour volumes of the ten wheats. Each bar represents the mean of 60 replications.

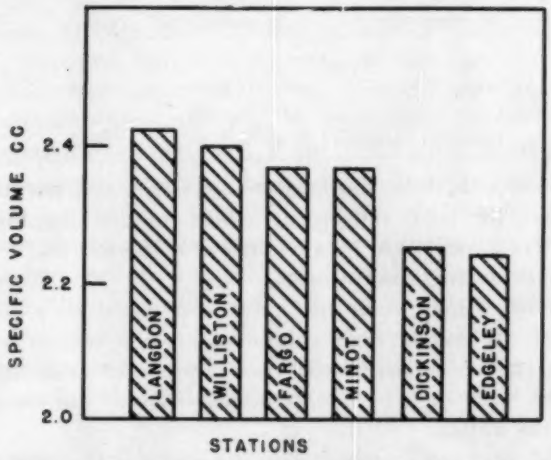

Fig. 3. Specific flour volumes of the wheats as influenced by locations of growth. Each bar represents the mean of 100 replications.

all stages during milling. Mida would also require slightly more volume, although the additional volume occupied by Mida should not cause any difficulty in the milling process. This variety has apparently been found satisfactory by the milling industry in other years when

rust did not reduce the test weight of this variety as drastically as in 1954. The flour volumes for Willet and Lee were larger for the experimentally milled samples than for the semicommercially milled, but there was little difference for Mida. However, since the two groups of wheats were not grown comparably the results cannot be considered as representative of differences caused by milling procedures. The flour volumes of the three wheats were in the same order for both milling methods, however.

The interaction (Table II) represents, in terms of flour specific volume, the differential effect of location of growth on the variety. This effect is very significant, so that the varieties did not react similarly at the different stations as judged by specific volume. No relation was apparent between specific flour volume and test weight, wheat protein content, flour ash content, or baking quality.

Micro-milled flours varied in volume in the same manner as the semicommercial and the Allis flours. In Table III are the flour volumes for three hybrid wheats from the nursery which had different kernel characteristics and milling properties.

TABLE III
FLOUR VOLUMES FOR THREE HYBRID WHEATS

Sample	Kernel Appearance	Milling Properties	Flour Volume	Specific Flour Volume
			ml.	*ml/g*
Kenya AC	White	Soft and fluffy	220.6	2.94
Composite	Pale	Soft and fluffy	214.4	2.86
ND 1	Dark and vitreous	Normal	162.3	2.16

The wheat with dark, hard, vitreous kernels and normal milling characteristics for hard red spring wheat yielded the lowest flour volume. There appeared to be a tendency for the soft and fluffy flours to be lower in baking absorption.

The results of these tests indicate that it might be a good policy to determine the specific flour volume of all new wheats before they are released. If the variety should occupy a considerable acreage and it was undesirably high in flour volume, it could quite easily cause trouble in the mills.

It should be kept in mind that these tests were made on pure varieties. This condition almost never occurs in commercial milling and the differences would therefore be reduced.

Literature Cited

1. HARRIS, R. H., and SCOTT, G. M. Particle size distribution in experimentally milled flours from wheat varieties of various quality. Northwestern Miller (Sec. 2) 243 (6): 1a, 10a–13a (Feb. 8, 1955).

THE ACTION OF WHEAT AMYLASES ON STARCH UNDER CONDITIONS OF TIME AND TEMPERATURE AS THEY EXIST DURING BAKING[1]

C. C. WALDEN[2]

ABSTRACT

Crumb temperatures during baking, at distances inside a loaf of bread exceeding ¾ in., were found to increase with time in a uniform manner and were unaffected by such variables as loaf size, pan shape, and variation in normal baking formula. Starch-amylase systems containing alpha- and beta-amylases alone and in combination were subjected to thermal treatments from 30° to 98°C. designed to duplicate conditions inside a loaf of bread during baking. Starch conversion (as maltose) and enzyme denaturation were determined for each system at increasing times of the thermal treatment. After heat treatment, D-glucose and maltose were determined in those systems which contained alpha-amylase initially. From these data the average degree of polymerization of the residual dextrins was calculated. Results showed that a high degree of starch conversion was produced by the action of alpha-amylase and a smaller but definite amount when beta-amylase was present. In combination, larger quantities of maltose were produced and the average chain length of residual dextrins was reduced.

The role of the amylolytic enzymes in the baking process has been traditionally associated with the production of sugar to support yeast fermentation. More recently, as the role of starch in bread quality has unfolded, attention has been centered on the function of amylases during the actual baking period. Kozmin (17) first recognized the effect of amylases on quality and postulated that excessive dextrinization was responsible for sticky crumbs found when flours milled from highly germinated wheats were baked into bread. Subsequent investigation (15) suggested a beneficial role for alpha-amylase in breadmaking, apart from producing sugar for fermentation. Difficulties in distinguishing between the effects of amylases and proteases, both present in malt supplements, were overcome by preparation of amylases free from proteinases (4, 19). Baking improvement was then shown to result from the addition of moderate levels of alpha-amylase to the baking formula (11, 12, 18, 20), whereas high levels produced sticky crumbs.

Our present qualitative knowledge of the action of alpha-amylase during baking is based largely on the parallelism with the action of the enzyme on gelatinized starch fractions. Here the reaction goes in

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two stages; the first resulting in the production of dextrins and limited quantities of D-glucose and maltose, the second being a slower conversion of dextrins to D-glucose and maltose. The slower rate in the second stage of the reaction is caused by the lesser affinity of alpha-amylase for the dextrins as a substrate. Over the time intervals in baking, it is probable that this second stage of alpha-amylase action is unimportant.

The effect of the rate of temperature increase, on both starch conversion and amylase denaturation, inside a loaf during baking, makes difficult the projection of data obtained at any given temperature or temperatures. Reports on the action of amylases on starch under conditions as they exist during baking are meager. Indeed, relatively few publications are available concerning the conditions of time and temperature inside a loaf of bread during baking (1, 2, 3, 8). It was considered advisable, therefore, to examine amylase-starch systems whose time-temperature conditions duplicated those found inside a loaf of bread during baking.

Materials and Methods

Starch. Starch was prepared from a commercial top patent flour, milled from Western Canadian hard red spring wheat, by the procedure of Walden and McConnell (29).

Alpha-Amylase. Purified but uncrystallized alpha-amylase was prepared by the method of Schwimmer and Balls (25), as adapted to a malted wheat flour source in these laboratories.

Beta-Amylase. Beta-amylase was prepared by the method of Walden (28) from an unsupplemented straight grade flour, milled from Western Canadian hard red spring wheat.

Heat-Treatment Bath. A stainless steel 4-liter beaker, containing 1 liter of water and 415 g. calcium chloride, was used as a heat-treatment bath. A 600-ml. low-form beaker containing 10 g. of air-dried starch and 200 ml. of water was immersed in the bath to the liquid level. Two burners were used for heating the bath, and the starch slurry and bath liquid were stirred mechanically. A heating curve for the starch slurry is shown in Fig. 1.

Heat Treatment. Alpha- and beta-amylases in aqueous solution were added to a starch slurry containing 10 g. air-dried starch, the final liquid volume being increased to 200 ml. with water. During heat-treatment, 3-ml. samples were removed at 1-minute intervals, when temperature was also read.

Alpha-Amylase Activity. The samples were placed immediately in 47 ml. of 0.149% calcium sulfate solution held at 0°C. and were then

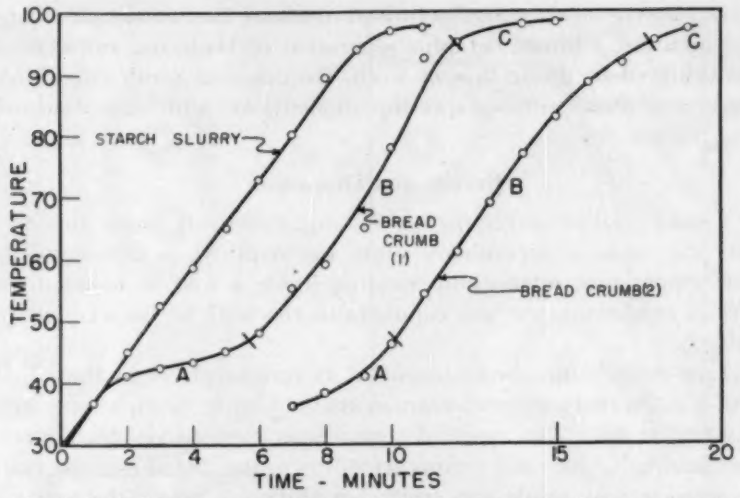

Fig. 1. Typical time-temperature curves for bread crumb during baking and a similar curve for a starch slurry heated under arbitrary conditions designed to duplicate parts B of the curves for bread crumb. Bread crumb: (1) 1 inch inside the crust; (2) center of a 1-lb. loaf.

centrifuged free of insoluble starch. Alpha-amylase activities were measured by the method of Sandstedt, Kneen, and Blish (24) as modified for spectrophotometric evaluation by Walden, Spencer, and Geddes (31). Where necessary, micro determinations were made by the method of Stone (26) and evaluated spectrophotometrically (31). Blank determinations showed that sample dilutions were sufficiently high that starch products carried over in the samples did not affect the determinations.

Beta-Amylase Activity. The samples were placed in 47 ml. of distilled water held at 0°C., centrifuged free of insoluble starch, and then held 48 hours at 30°C. to complete beta-amylolysis. Beta-amylase activity was determined by the method of Kneen and Sandstedt (14). Where alpha-amylase was present, the saccharifying equivalence of alpha-amylase activity was determined first and the beta-amylase activity then calculated by difference.

Starch Conversion. The samples were placed in a mixture of 20 ml. of diluted acetate buffer (14, 24), 20 ml. of 1% sulfuric acid, and 7 ml. of distilled water. Reducing sugars were determined as maltose by ferricyanide reduction (14) and calculated as percent starch conversion.

Chromatographic Separation and Quantitative Estimation of D-Glucose and Maltose. The portion of the starch-amylase system remaining after each heat treatment was cooled and stored in the refrigerator under thymol and toluene. Before chromatographic separa-

tion, reducing sugars were determined to ensure that no sample change had occurred. Chromatographic separation of D-glucose and maltose was achieved by the technic of Koch, Geddes, and Smith (16). These sugars were then estimated spectrophotometrically with phenol-sulfuric acid reagent (5).

Results and Discussion

Crumb Temperatures During Baking. Before the main investigation was made, a preliminary study was required to determine the time-temperature relationship existing inside a loaf of bread under various conditions, and this supplements the work of Barackman and Bell (2).

Loaf temperatures were measured at various places in the crumb with a calibrated copper-constantan thermocouple. Oven temperature was 230° ± 3°C. The types of time-temperature curves encountered may be divided into two groups according to the distance of the point of measurement inside the crust. For distances inside the loaf not exceeding ¾ in., the rate of temperature rise varied inversely with distance from the dough skin. Final temperatures varied similarly from 100°C. at ¾ in. inside the crust to 195°C. on the outside of the crust. At distances inside the crust exceeding ¾ in., certain similarities existed between various time-temperature curves; typical curves are shown in Fig. 1 and have been arbitrarily divided into three parts. Parts A, extending from 30° to 47°C. (Fig. 1), show a relatively slow temperature rise. Parts A of curves derived from various locations in the crumb extend over a time interval depending directly on their distance from the loaf surface. Parts B, from 47° to 95°C., are similar for the various curves, being displaced to shorter or longer time intervals depending on the distance of the point of measurement from the crust surface. Parts C, extending from 95° to about 98°C., are similar for all time-temperature curves excepting the length of time at about 98°C. Variables examined, such as loaf size, pan shape, and differing formulas, had no measurable effect.

For studies of amylase-starch systems, part B of this curve is the most important. Below 55°C. starch is not gelatinized and amylase action is insignificant. Above 95°C., thermal denaturation of the malt amylases is complete. The heat treatment bath described above was set up to duplicate experimentally parts B of the crumb temperature curve.

Alpha-Amylase. For starch alpha-amylase systems subjected to the same conditions of time and temperature as bread crumb during baking, the amount of starch conversion and enzyme denaturation is given

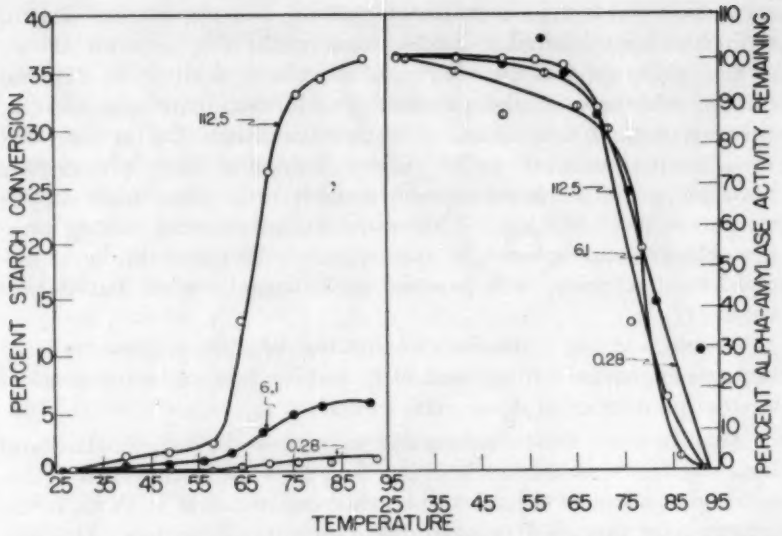

Fig. 2. Starch conversion (as maltose) and thermal denaturation of alpha-amylase in starch-alpha-amylase systems subjected to conditions of time and temperature as they exist inside a loaf during baking. Numerical values are initial alpha-amylase units per g. of starch. Commercial flours normally contain the equivalent of 0.28 units per g.

in Fig. 2. Starch was rapidly attacked over the approximate temperature range of 58°–78°C., while above 78°C. the rate of starch conversion declined owing to enzyme denaturation. Starch conversion was small for 0.28 alpha-amylase units per g. For 6.1 units per g. the final amount of conversion was 6.1%, and the average dextrin chain was composed of 60 D-glucose units (Table I). For 112.5 units per g., 36.5%

TABLE I

D-GLUCOSE, MALTOSE CONTENTS, AND AVERAGE DEXTRIN LENGTH OF STARCH HYDROLYSATES FOLLOWING ACTION BY VARIOUS AMOUNTS OF ALPHA- AND BETA-AMYLASES UNDER CONDITIONS OF TIME AND TEMPERATURE AS THEY EXIST INSIDE A LOAF OF BREAD DURING BAKING

Initial Amylase Contents		Starch Conversion	Glucose	Maltose	Average D.P.[a] of Dextrins
Units α-amylase per g. of starch	Units β-amylase per g. of starch				
		%	%	%	
6.1	0.0	6.1	0.2	2.5	60.0
112.5	0.0	36.5	2.9	10.0	8.2
5.6	11.2	16.2	0.9	14.4	very high
5.6	22.4	26.7	0.5	19.3	23.0

[a] The reducing power equivalent of the dextrins (starch present initially less D-glucose and maltose formed) corresponds to the total reducing power of the hydrolysate (starch conversion) less the reducing equivalent of the D-glucose and maltose. From this relationship, the average Degree of Polymerization has been calculated as numbers of D-glucose residues.

conversion was not proportionately greater, but the average dextrin chain had been reduced to 8.2 D-glucose units. The substrate affinity of malt alpha-amylase for dextrins of this size is small (9, 21, 22), and further additions of alpha-amylase should not materially increase amounts of starch conversion, under these conditions. On the basis that wheat flour contains 60 to 70% starch, a value of 0.28 alpha-amylase units per g. of starch corresponds roughly to a wheat malt supplementation level of 0.25%. This is normal commercial milling practice. Flours supplemented at approximately 20 times this level (6.1 alpha-amylase units) may produce sticky crumbs when baked into bread (12).

As shown in Fig. 2, thermal denaturation of alpha-amylase occurred between approximately 62° and 90°C. and the rate was not dependent on the quantity of alpha-amylase present.

Beta-Amylase. Starch conversion to maltose by beta-amylase and beta-amylase denaturation is shown in Fig. 3. Between starch gelatinization at about 58°C. and beta-amylase denaturation at about 72°C., beta-amylase produced considerable quantities of maltose. The rate of starch conversion above 72°C. fell to virtually zero corresponding to complete enzyme denaturation. Under the experimental conditions used here, higher levels of beta-amylase produced more maltose, but

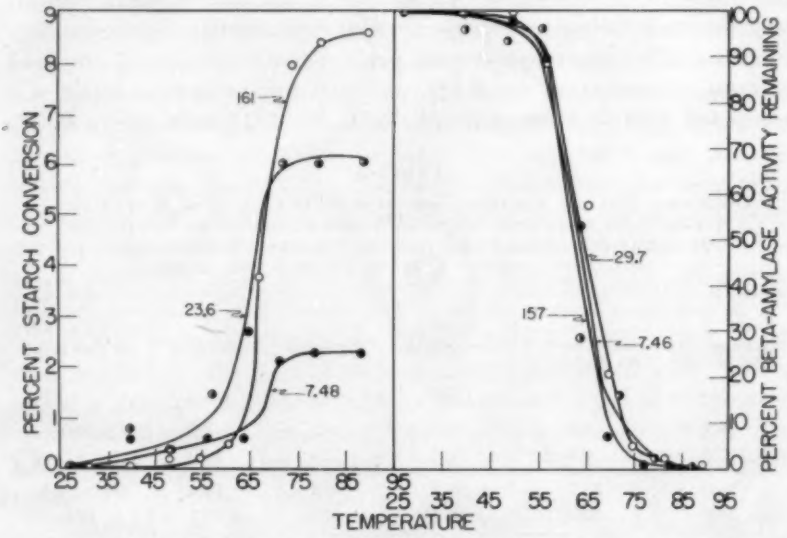

Fig. 3. Starch conversion (as maltose) and thermal denaturation of beta-amylase in starch-beta-amylase systems subjected to conditions of time and temperature as they exist inside a loaf during baking. Numerical values are initial beta-amylase units per g. of starch. Commercial flours normally contain the equivalent of 23.6 units per g.

not proportionately so. Total conversion fell far short of the theoretical limit of 65–66%. Since maltose, maltotriose, and beta-limit dextrin are the only products of starch conversion by beta-amylase (10, 13, 27), no chromatographic separations were attempted. On the basis that wheat flour contains 60 to 70% starch, 23.6 beta-amylase units per g. of starch correspond roughly to the beta-amylase activity of flours and doughs when sodium chloride is present (6, 30).

Thermal denaturation of beta-amylase occurred over the approximate temperature range of 54°–74°C. There was no indication that the rate of thermal denaturation depended on the quantity of beta-amylase present.

Combinations of Alpha- and Beta-Amylase. Figure 4 shows the starch conversion and enzyme denaturation in systems where alpha- and beta-amylases are acting jointly. The complementary effect of these two enzymes resulted in considerably greater starch conversion, at the same enzyme concentrations, than was achieved with either enzyme acting alone. For example, 5.6 alpha- plus 22.4 beta-amylase units produced 26.7% starch conversion compared to 6.1% for alpha-amylase (6.1 units) and 6.2% for beta-amylase (23.6 units) where the enzymes acted singly. In addition to the greater conversion measured as maltose, average dextrin chain length was reduced from 60 D-glucose units

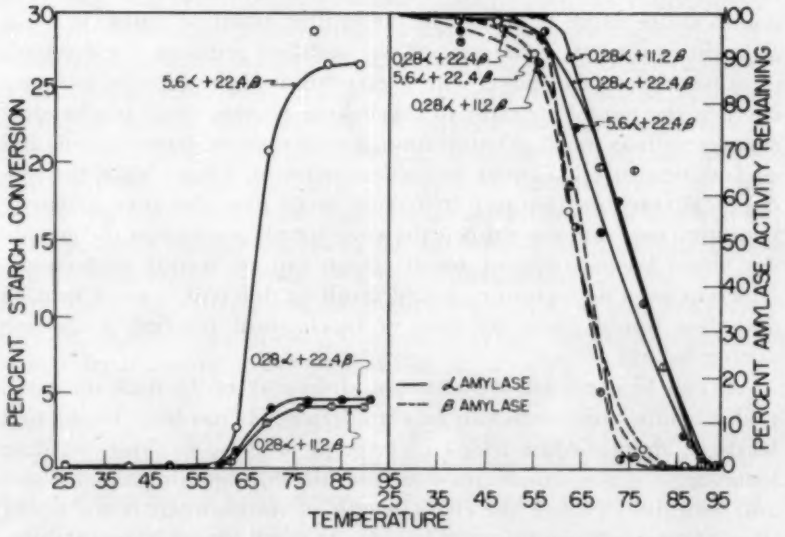

Fig. 4. Starch conversion (as maltose) and thermal denaturation of alpha- and beta-amylases in starch-amylase systems subjected to conditions of time and temperature as they exist inside a loaf of bread during baking. Numerical values are initial amylase units per g. of starch. Commercial flours normally contain the equivalent of 0.28 units of alpha- and 22.4 units of beta-amylase per g. of starch.

when alpha-amylase was acting singly, to 23 units when beta-amylase was also present (Table I). As previously stated, these levels of alpha- and beta-amylase correspond to levels in malt-supplemented flours which, on baking, yield loaves with sticky crumbs. Normal amylase levels in commercial flours correspond to 0.28 alpha- and 22.4 beta-amylase units per g. of starch. At this dosage alpha-amylase has had little effect on the amount of starch conversion or on the chain length of the residual dextrins.

Thermal denaturation of the combined amylases was the same as when the amylases were present singly.

General Discussion

Consideration of heat and moisture transfer inside a loaf of bread during baking suggests that crumb temperature changes with time should follow a uniform pattern. Under the influence of the external heat of the oven, heat is transferred to the inside of the loaf and moisture and other volatiles move out. The time interval necessary for these processes to establish themselves will depend on the distance of any particular portion of the crumb from the crust surface. Following the initiation of these processes, the crumb undergoes a rapid rise in temperature proportional to the difference between crumb and oven temperatures. As long as free moisture remains in the crumb the final crumb temperature is limited to the boiling point of water. In areas adjacent to the loaf surface where the moisture reservoir is exhausted, crust formation takes place. The steep portion of the time-temperature curve is the most important in the baking process, since biochemical changes such as starch gelatinization, gluten protein denaturation, and enzyme denaturation occur in this temperature range. Since the biochemical reactions resulting in baking occur over this time and temperature range and since this is the same for all portions of the crumb, the visual homogeneity of bread crumb can be readily understood. Different oven temperatures would result in different rates of heating and thus would affect the rate of biochemical reactions occurring during baking.

As may be expected, the action of alpha-amylase by itself on starch under baking conditions can be considerable. It has been found that levels of alpha-amylase which correspond to those in flours yielding loaves with sticky crumbs produce considerable quantities of maltose and materially reduce the chain length of starch when tested under time-temperature conditions of baking. At much higher levels of alpha-amylase, starch conversion may be considered to be complete, under the time conditions prevailing.

Acting alone, beta-amylase produces maltose from starch under oven conditions. High levels of beta-amylase, however, result in starch conversion still considerably below the theoretical yield. The effect of beta-amylase acting jointly with alpha-amylase is more pronounced. Much larger quantities of maltose are produced and the chain length of the residual dextrins is materially reduced. Since beta-amylase is present in normal sound flours in relatively large quantities, its effect must be taken into account. In comparing the effect during baking of alpha-amylases from different sources, not only must the beta-amylase content of the flour doughs be considered, but also any beta-amylase present in any particular malt alpha-amylase preparation. Beta-amylase may be eliminated from malt alpha-amylase preparations by suitable heat-treatment, but in a number of studies reported in the literature this has not been done.

Since beta-amylase is active during baking, excessive starch dextrinization which results in sticky crumbs in the resulting loaves is caused not merely by high alpha-amylase activity, but rather by an unbalance between the amylases in favor of alpha-amylase. In normal commercial flours, malt-supplemented or otherwise, beta-amylase normally present or produced during fermentation occurs in amounts sufficient that the principal product of starch conversion during baking is maltose. When alpha-amylase supplementation is materially increased, without similarly increasing the beta-amylase content, large quantities of dextrins are produced. However, if supplementation included beta-amylase, so that the two amylases remained in balance, larger quantities of maltose would be formed, without danger of excessive dextrinization. Presumably the quantity of maltose which could be produced in this manner would be governed only by the amount of unconverted starch necessary for the retention of loaf shape.

While the foregoing discussion has been based on the application of data from starch-amylase systems to the baking process, actual baking conditions may result in greater conversion of starch by the amylases, owing to the protective action of dough ingredients against thermal denaturation of the amylases in the oven (7, 20, 23).

Acknowledgment

The author wishes to acknowledge the technical assistance of Mr. D. R. Grant throughout the experimental studies and the financial assistance accorded by the Associate Committee on Grain Research of the National Research Council of Canada.

Literature Cited

1. Bailey, C. H., and Munz, E. The march of expansion and temperature in baking bread. Cereal Chem. 15: 413–418 (1938).

2. Barackman, R. A., and Bell, R. N. Temperatures attained in baking products. Cereal Chem. **15:** 841–845 (1938).

3. Berg, I. A. The cooling of bread. Part I. Baking Technol. **5:** 205–208 (1926).

4. Dirks, B. M., and Miller, B. S. Inactivation and removal of proteolytic enzymes from amylolytic supplements. Cereal Chem. **26:** 98–109 (1949).

5. Dubois, M., Gilles, K., Hamilton, J. K., Rebers, P. A., and Smith, F. A colorimetric method for the determination of sugars. Nature **168:** 167 (1951).

6. Erlich, V. L., and Burkert, G. M. Beta-amylase activities of barley, wheat and rye. Cereal Chem. **27:** 423–437 (1950).

7. Falunina, Z. F., and Popadich, I. A. [In Russian.] Thermal stabilities of amylases in heated wheat dough. Doklady Akad. Nauk S.S.S.R. **78** (N.S.): 103–106 (1951). Translation of title from Chem. Abstr. **45:** 8669g (1951).

8. Hashitani, Y., and Sako, T. On the vitamin B content of bread. Cereal Chem. **9:** 107–117 (1932).

9. Hopkins, R. H. The actions of the amylases. *In* Advances in enzymology, ed. by E. F. Nord, Vol. 6, pp. 389–414. Interscience: New York (1946).

10. Hopkins, R. H., Jelinek, B., and Harrison, L. E. The action of β-amylase on potato amylose. Biochem. J. **43:** 32–38 (1948).

11. Johnson, J. A., and Miller, B. S. High levels of alpha-amylase in baking. I. Evaluation of the effect of alpha-amylase from various sources. Cereal Chem. **25:** 168–178 (1948).

12. Johnson, J. A., and Miller, B. S. Studies on the role of alpha-amylase and proteinase in breadmaking. Cereal Chem. **26:** 371–383 (1949).

13. Kerr, R. W., and Cleveland, F. C. The action of crystalline β-amylase on corn crystalline amylose. J. Am. Chem. Soc. **73:** 2421–2424 (1951).

14. Kneen, E., and Sandstedt, R. M. Beta-amylase activity and its determination in germinated and ungerminated cereals. Cereal Chem. **18:** 237–252 (1941).

15. Kneen, E., and Sandstedt, R. M. The evaluation of malt for use as a flour supplement. Cereal Chem. **19:** 181–195 (1942).

16. Koch, R. B., Geddes, W. F., and Smith, F. The carbohydrates of *Gramineae*. I. The sugars of the flour of wheat (*Triticum vulgare*). Cereal Chem. **28:** 424–430 (1951).

17. Kozmin, Natalie. Biochemical characteristics of dough and bread from sprouted grain. Cereal Chem. **10:** 420–436 (1933).

18. Miller, B. S., and Johnson, J. A. High levels of alpha-amylase in baking. II. Proteolysis in straight and sponge doughs. Cereal Chem. **25:** 178–190 (1948).

19. Miller, B. S., and Johnson, J. A. Differential stability of alpha-amylase and proteinase. Cereal Chem. **26:** 359–371 (1949).

20. Miller, B. S., Johnson, J. A., and Palmer, D. L. A comparison of cereal, fungal, and bacterial alpha-amylases as supplements for breadmaking. Food Technol. **7:** 38–42 (1953).

21. Myrbäck, K. Über die Wirkungsweise der α-Malzamylase. Arkiv Kemi, Mineral. Geol. **26A:** No. 7, pp. 1–9 (1949). Chem. Abstr. **44:** 7364a (1950).

22. Myrbäck, K., and Sihlbom, Esther. Wirkung der Malzamylasen auf niedrig molekulare Hydrolyseprodukte der Stärke. Arkiv för Kemi **1:** 1–16 (1949). Chem. Abstr. **43:** 5219a (1949).

23. Pronin, S. I., and Petrova, I. S. [In Russian.] Thermostability of amylases in dough during baking. Doklady Akad. Nauk S.S.S.R. **85** (N.S.): 855–858 (1952). Translation of title from Chem. Abstr. **47:** 3894e (1953).

24. Sandstedt, R. M., Kneen, E., and Blish, M. J. A standardized Wohlgemuth procedure for alpha-amylase activity. Cereal Chem. **16:** 712–723 (1939).

25. Schwimmer, S., and Balls, A. K. Isolation and properties of crystalline alpha-amylase from germinated barley. J. Biol. Chem. **179:** 1063–1074 (1949).

26. Stone, I. A sensitive method for the determination of alpha-amylase activity of flour. Cereal Chem. **29:** 228–234 (1952).

27. SWANSON, MARJORIE A. Studies on the structure of polysaccharides. II. Degradation of polysaccharides by enzymes. J. Biol. Chem. **172:** 805–814 (1948).

28. WALDEN, C. C. Purification of wheat alpha- and beta-amylases. Thesis submitted to the University of Minnesota, February 1954.

29. WALDEN, C. C., and MCCONNELL, W. B. Studies on technics for reconstituting flours. Cereal Chem. **32:** 227–236 (1955).

30. WALDEN, C. C., SPENCER, E. Y., and GEDDES, W. F. Release of beta-amylase in flour suspensions and doughs. (In state of publication.)

31. WALDEN, C. C., SPENCER, E. Y., and GEDDES, W. F. Investigations on a spectrophotometric method for the determination of alpha-amylase activity. (In state of publication.)

BOOK REVIEWS

Statistical Analysis in Chemistry and the Chemical Industry, by Carl A. Bennett and Norman L. Franklin. John Wiley & Sons, Inc., New York, 1954. Price $8.

This book should appeal to its intended audience of chemists and chemical engineers, for the usual biological examples are avoided. The examples included have been selected with care to show the many different statistical methods that can be applied to chemistry and the many different branches of chemistry to which statistics can be applied.

It is not, however, a cookbook. Each topic is covered completely, starting with development of the underlying theory and the derivation of the required formulas, and ending with illustrative examples and computational procedures. The same approach—first providing the theory so that the reader can judge the applicability of the method—accounts for the order of presentation of the topics.

The first five chapters present the basic principles of statistical inference. Chapter 2, on descriptive statistics, explains and clearly defines many of the terms that confuse a person with an awakening interest in the subject. Chapters 3 and 4, on probability and mathematical machinery, are treated comprehensively as evidenced by the inclusion of moment-generating functions and semiinvariants. Throughout these early chapters emphasis is placed on the difference between sample and population statistics and the purpose and need of randomization.

The last six chapters are devoted to those methods that have been of greatest use in industry. The relative importance of analysis of variance and design of experiments is apparent from the fact that these two chapters cover 159 and 122 pages respectively. The various mathematical models for analysis of variance are considered in detail. Estimated values of mean squares and the resulting tests of significance are presented clearly and correctly, which unfortunately has not always been done.

In spite of the length of the chapter on design of experiments, some of the newer technics, such as steepest ascent and response surface designs, have been given only a paragraph. It must be added, however, that this is a rapidly developing science and the book was a long time in the making, having been started in 1950. Treatment of factorial experiments is very thorough, along with that of confounding and fractional replication.

In the references, several fine papers by K. A. Brownlee *et al.* have been omitted, but on the whole there are more references than usual, and this completeness is characteristic of the book. Unfortunately, however, numerous incorrect notations will temporarily confuse even the professional statistician. A graduate chemist or engineer could acquire a sound understanding of the theory and use of modern statistical methods from the book, provided he spends considerable time and effort studying the contents. A true appreciation of what statistics can do for him will then follow as he applies some of these methods to his own experimental work.

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Soil Fertility, by C. E. Millar. 436 pp. John Wiley and Sons, New York, 1955. Price $6.75.

This book is intended as a text for a course in soil fertility and as a reference for soil management courses. The author is Professor Emeritus of Soil Science, Michigan State College.

The first four chapters are preliminary to the main theme of the book. The first is an interesting but brief review of the development of agriculture; the second discusses some plant-physiological principles; the third and fourth deal with the soil solution and colloids, respectively.

The major and minor elements, as well as some "nonessential nutrients," are

given detailed consideration. There are chapters that deal with liming, organic matter, animal and green manures, commercial fertilizers, rotations, and activities of soil organisms. There is a chapter on soil deficiencies and determination of nutrient needs of crops.

One of the most interesting and valuable chapters is the one that summarizes old field experiments. Here the author presents some of the history and results from experiments at Rothamsted, England, that were begun over 100 years ago, and experiments at eight state experiment stations in the United States that have been carried on continuously for 55 to 75 years. This summary and the references given are a valuable source for review of these important experiments.

It is unfortunate that the contribution of soil physical properties to productivity is not clearly expressed. The four chapters dealing with organic matter, animal and green manures, rotations, residues, and composts largely ignore the relationship of these to soil structure and its consequent effect on productivity, placing the emphasis rather on the importance of organic manures and residues as sources of nutrients. Furthermore, it would seem an improvement if these chapters were not separated from each other in the sequence.

This book is well documented. Numerous references are cited in footnotes, and there is a list for further reading at the close of each chapter. The data presented and accessibility of the references add much to the value of the book.

The book will be of value and interest not only to students of soils but to a much wider audience of persons who wish a discussion of fertilizer usage and needs of crops.

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Grain Crops, by Harold K. Wilson. 2nd ed., 395 pp. McGraw-Hill Book Company, Inc., New York, 1955. Price $6.50.

This book, designed primarily as a beginning text for students of grain crops, is an expansion of the earlier book by the same author. It is fully revised, and written from a background of many years of successful teaching experience and observations. Selection, arrangement, and presentation of material are excellent.

The first and second chapters, dealing with the significance of plants to life generally, the cycle of plant growth, the effect of environment and crop adaptation, and plant classification seek to inspire an early appreciation of the many complex phases of plant life and relationships. A more detailed discussion follows of each of the several grain crops and of their distribution and production throughout the world and in the United States.

The advantages of good rotation and the principles upon which a cropping program may be planned precede a discussion of soils and soil types, maintenance of soil fertility, and fertilizer requirements. Soil conditioning and soil conservation, through better control of erosion, are treated briefly. "Culture of grain crops" includes choice of seedbeds, seedbed preparation, time and depth of plowing, tillage, and what each of these operations seeks to accomplish; seed treatment, rates and time of sowing, harvesting and storing of the grain. Data supporting prescribed practices, refreshingly up to date and drawn from a wide area, illustrate rather fully the principles involved.

Three chapters cover special problems of production, including weed competition, plant diseases and insect pests. Loss from weeds alone is estimated to be in excess of 3 billion dollars annually. Weed control measures such as prevention, tillage, competing crops and chemicals, and the use of the newer selective herbicides and their advantages and limitations are discussed.

The chapter on plant diseases is designed to be helpful to the beginning student. General principles of disease control include prevention, seed treatment, and breeding for disease resistance. Diseases of wheat are given specific attention because of their major importance: stem rust, leaf rust, covered smut, loose smut, and scab. Similarly discussed are the major diseases of the other grain crops.

The next several chapters treat more specifically of each of the grain crops, wheat and rye, oats, barley, flax, rice, buckwheat, millet, soybeans, corn (including sweet corn and popcorn), and grain sorghums. For each crop there is a botanical

classification, a description of market classes, varieties, crop utilization, and special features of culture and handling not previously discussed. Harvesting and storage of corn, including corn for grain and corn for silage, receive attention in another chapter. Not overlooked are marketing, marketing processes, the place of the local elevator, terminal elevators, brokerage or commission houses, grain inspection services, and finally the selling of grain.

The final chapter discusses crop improvement – the need for it, and methods by which it can be accomplished. Hybridization, offering the greatest improvement through development of new varieties and hybrids, is discussed at some length. The importance of maintaining quality and purity of seed, and to this end the place of crop improvement associations and seed certifying agencies, are included.

The book is well written, well illustrated and up to date. Some sections may appear too brief or too general, but selected references permit of expansion and added emphasis. Teachers will find this a very good and useful text.

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Methods for Evaluation of Nutritional Adequacy and Status. Edited by H. Spector, M. S. Peterson, and T. E. Friedemann; vii + 313 pp. Quartermaster Food and Container Institute for the Armed Forces, Chicago, 1954. Copies are available gratis from the Institute, 1819 W. Pershing Road, Chicago 9, Ill.

Feeding large groups of military and frequently civilian populations is the prime responsibility of the Quartermaster Corps, and with this task falls the added responsibility, which is shared by the Office of the Surgeon General, of ensuring that the food provided is nutritionally adequate. It was for the purpose of bringing together the most authoritative and most recent information on the methods for evaluating nutritional adequacy that a symposium on this subject was sponsored by these two military agencies. The conference was held under the auspices of the Committee on Foods, Advisory Board on Quartermaster Research and Development, National Research Council.

Many of the leading nutritionists of this country participated in this symposium, which was divided into six sections. Five were for the evaluation of: (1) protein, (2) vitamin, and (3) mineral adequacy; (4) of military rations by animal experimentation; and (5) of the nutritional status of populations. The sixth was a round-table discussion on body composition. It is evident that a broad spectrum of subjects was covered, and this diversity of information served to emphasize the many complex factors involved in the relationship of food composition to body composition and function.

The fact that this book is paper-bound should not belie its value as a source of information to anyone interested in the fundamental and practical aspects of human nutrition.

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Experimental Cookery, by Belle Lowe. 4th ed., 573 pp. John Wiley & Sons, Inc., New York, 1955. Price $7.50.

The author has done an excellent job of bringing the book up to date on developments in the food technology field during the twelve years since publication of the third edition.

A new chapter in the fourth edition, entitled "Food Acceptability," takes the place of a chapter on food preservation. Much of the old material has been included in other chapters in the new edition. With this exception, chapters in the previous edition are retained, though rewritten and brought up to date.

The first chapter deals with the importance of colloid chemistry in preparation of foods. A chapter on food acceptability and methods of evaluating food products

follows. The next six cover such topics and products as sugar cookery, frozen foods, fruits and vegetables, pectic substances, gelatin, meats, and poultry. In another section on emulsions, milk and milk products, and eggs, the subject matter deals with products in which either emulsions or the ingredients covered play a major role. At this point in the book, a student of food preparation is well prepared for the next two chapters on starch, flour and bread, and batters and doughs. The final chapter, on fats and oils, completes a rather well-rounded discussion of the important ingredients used in preparation of foods.

One of the book's strong points is its good documentation. In addition to plentiful references, each chapter has an excellent bibliography and many of these are subdivided according to subject matter discussed.

As in previous editions, the main purpose of this book is to explain changes that occur during preparation of a food product, in chemical and physical terms. Not only is it an excellent textbook, with laboratory experiments covering such matter discussed in each chapter, but it is also an excellent reference book on foods and food products.

J. Robert Roach
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SUGGESTIONS TO AUTHORS

General. Authors will find the last volume of *Cereal Chemistry* a useful guide to acceptable arrangements and styling of papers. "On Writing Scientific Papers for Cereal Chemistry" (*Trans. Am. Assoc. Cereal Chem.* 6:1–22. 1948) amplifies the following notes.

Authors should submit two copies of the manuscript, typed double spaced with wide margins on 8½ by 11 inch white paper, and all original drawings or photographs for figures. If possible, one set of photographs of figures should also be submitted. Originals can then be held to prevent damage, and the photographs can be sent to reviewers.

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Use names, not formulas, for text references to chemical compounds. Use plural verbs with quantities (6.9 g. were). Figures are used before unit abbreviations (3 ml.), and % rather than "per cent" is used following figures. All units are abbreviated and followed by periods, except units of time, which are spelled out. Repeat the degree sign (5°–10°C.). Place 0 before the decimal point for correlation coefficients ($r = 0.95$). Use * to mark statistics that exceed the 5% level and ** for those that exceed the 1% level; footnotes explaining this convention are no longer required. Type fractions on one line if possible, e.g., $A/(B + C)$. Use lower case for farinograph, mixogram, etc., unless used with a proper name, i.e., Brabender Farinograph. When in doubt about a point that occurs frequently, consult the Style Manual or the Dictionary.

For more detailed information on manuscript preparation see
Cereal Chem. **30:** 351–352 (1953).

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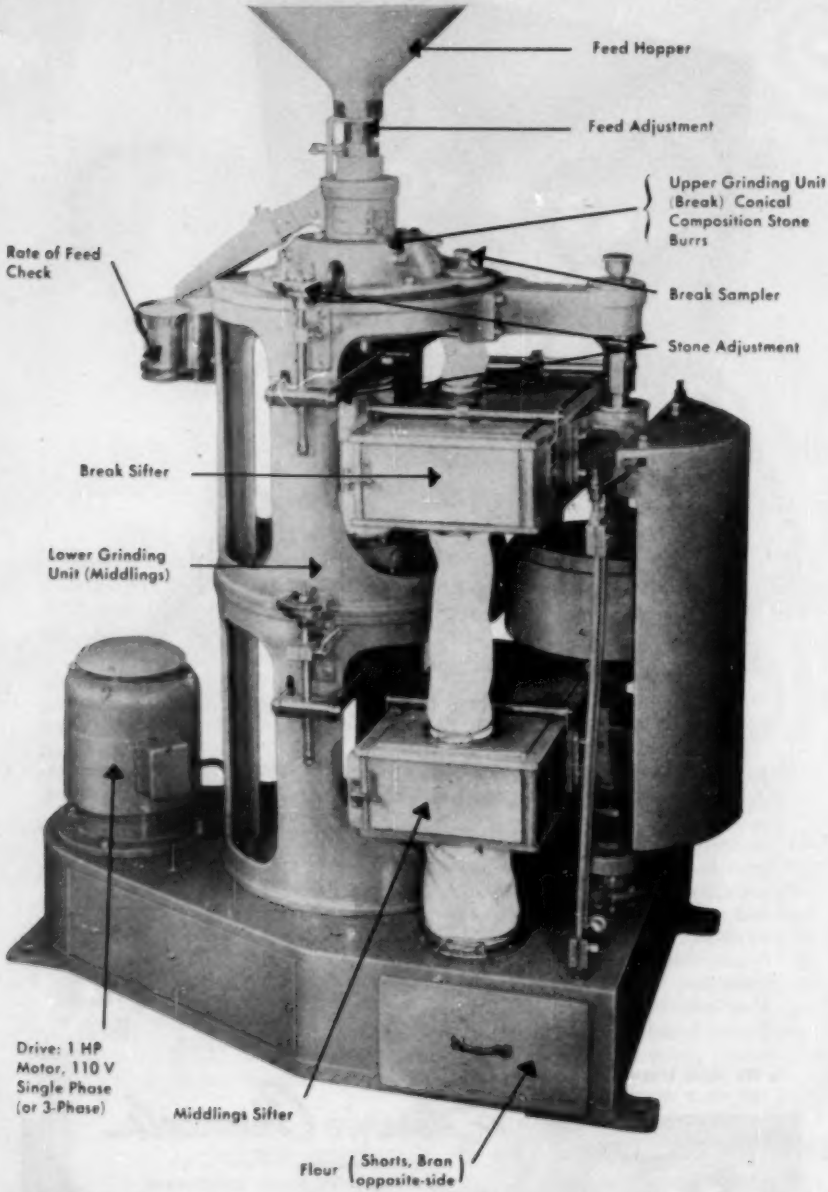

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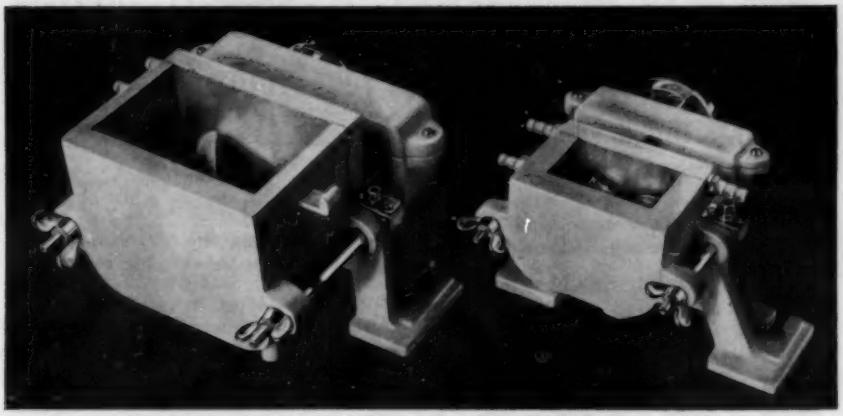

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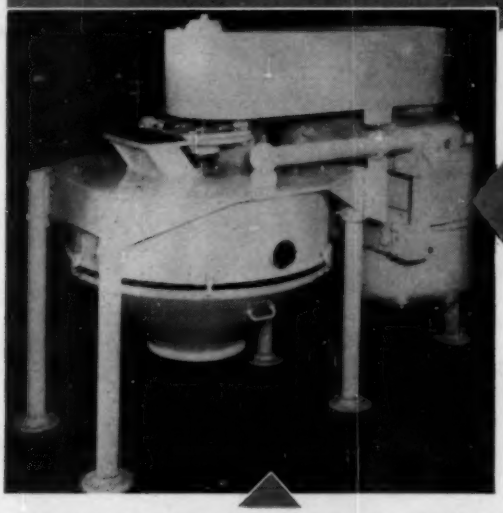

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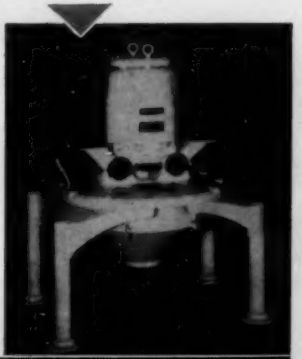

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